The Journal of LABORATORY and CLINICAL MEDICINE

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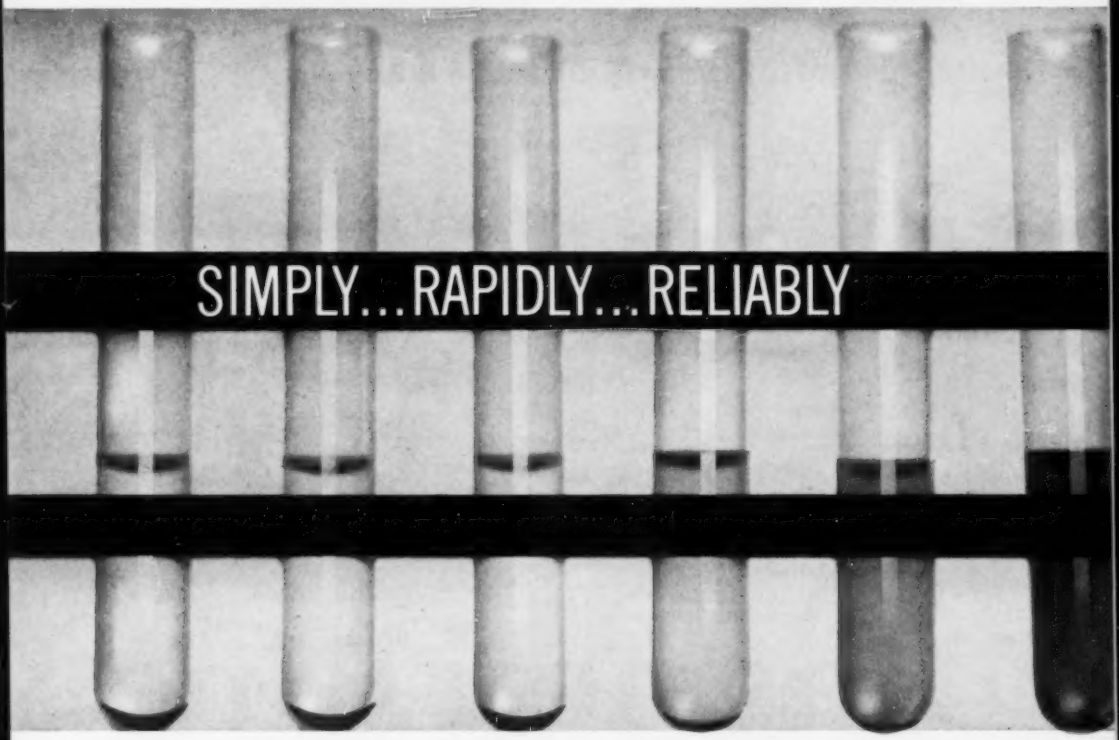

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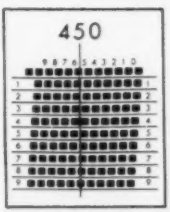

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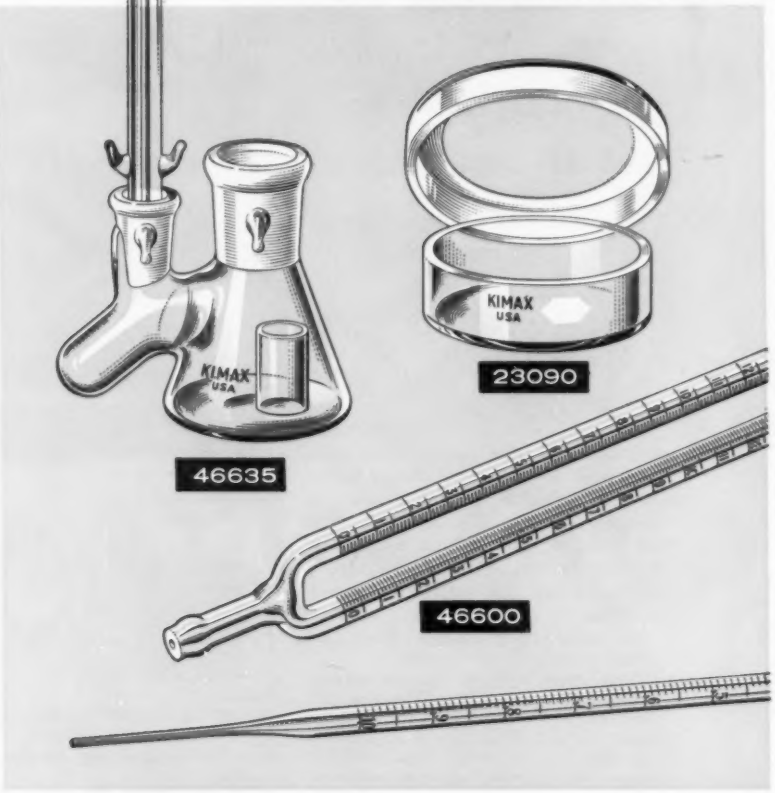

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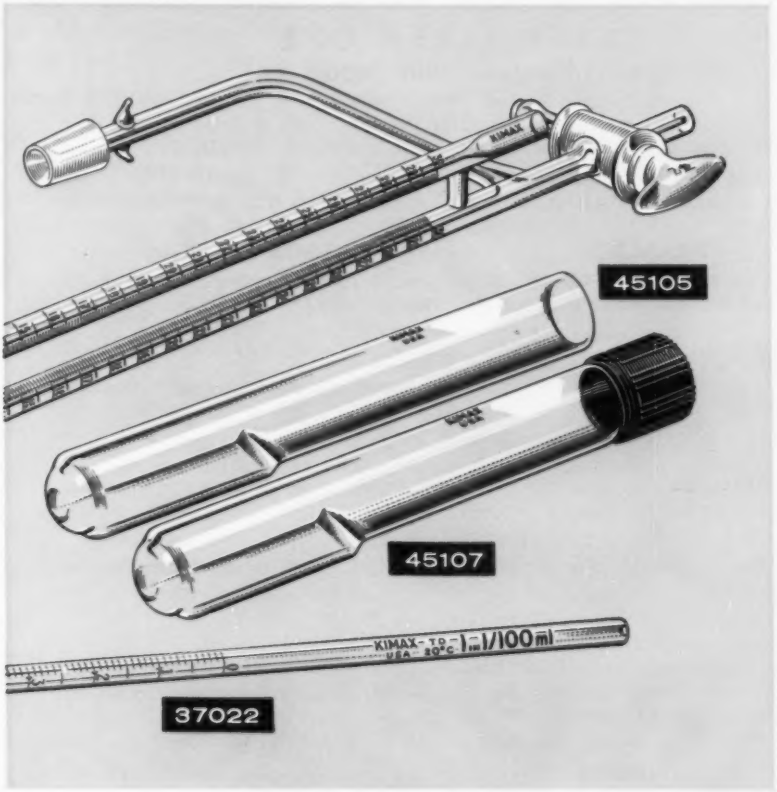

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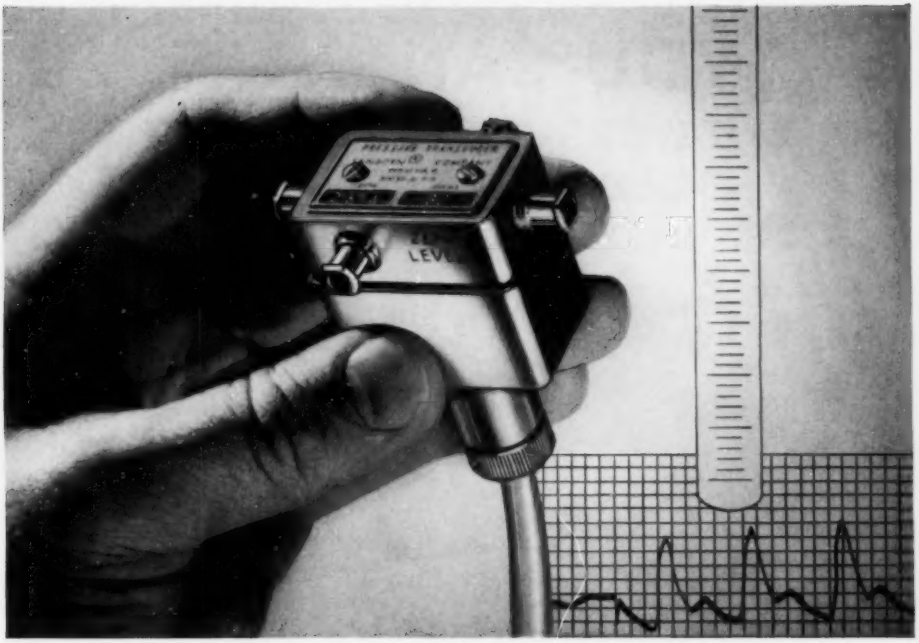

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1. Schiff, F. and Sasaki, H.: Zschr. Immunforsch. 77: 129-139, 1932.
2. Grubb, R.: Nature 162: 933, 1948.
3. Schiff, F.: Zschr. Immunforsch. 82: 302-310, 1934.
4. McNeil, C., et al: Am. J. Clin. Path. 28(2): 145-151, (Aug.) 1957.
5. McNeil, C., et al: Am. J. Clin. Path. 28(5): 469-480, (Nov.) 1957.

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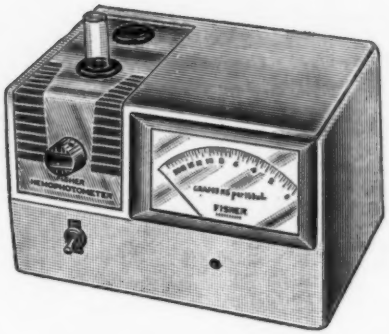

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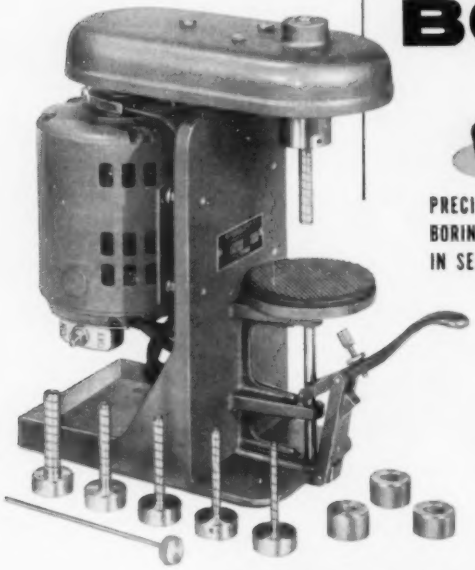

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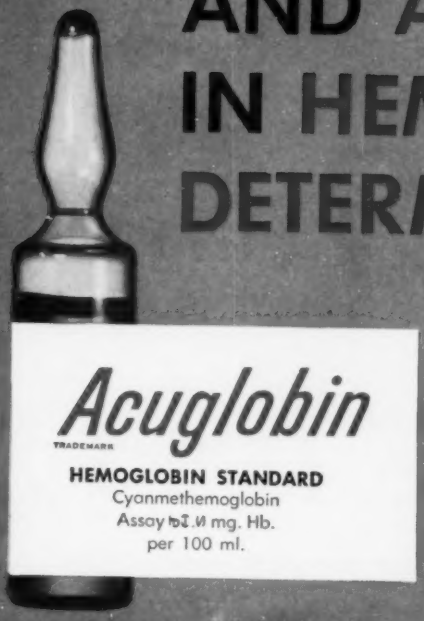

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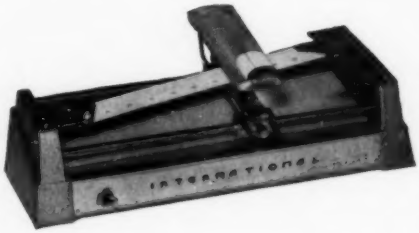

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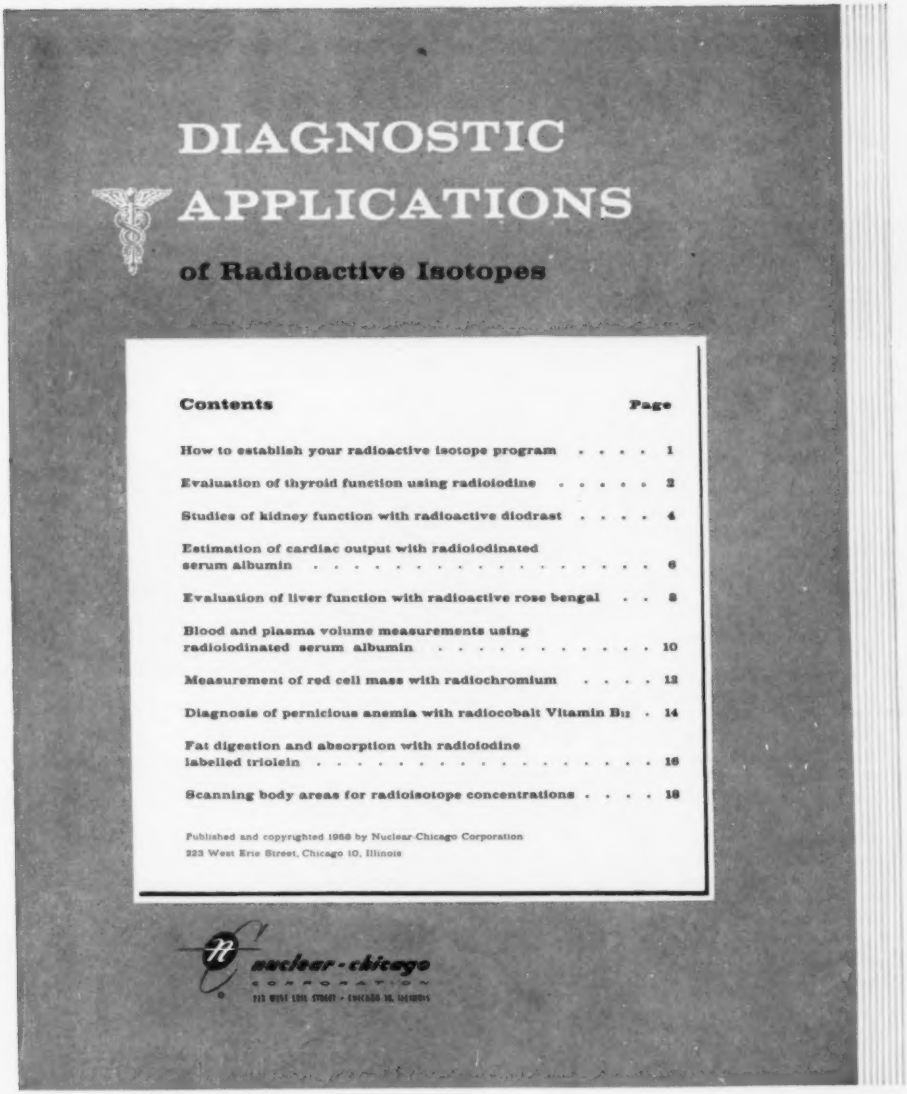
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* The Chloridometer is produced according to the design of Ernest Cotlove, Hillary V. Trantham, and Robert L. Bowman, of the Laboratory of Kidney and Electrolyte Metabolism and the Laboratory of Technical Development, National Heart Institute, Bethesda, Maryland. Special acknowledgments and thanks are due to Dr. Cotlove for his patient cooperation and helpfulness in putting Chloridometers installed in his laboratory to the most severe tests and for his suggestions toward refinements for future production.

Ref.: Cotlove, E.; Trantham, H. V.; and Bowman, R. L.: An Instrument and Method for Automatic, Rapid, Accurate, and Sensitive Titration of Chloride in Biological Samples, J. LAB. & CLIN. MED.: *Vol. 50, No. 3, pp. 358-371, 1958.* (Copies obtainable on request from Laboratory Glass & Instruments Corp.)

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Glucosamine

Because of an item we read in the *Wall Street Journal* recently, a copy or abstract of which we refuse to supply, we predict that this year will see an upsurge of interest in the biochemistry of glucosamine.

This amino sugar is easy to prepare. The classical starting material is chitin, and the essence of the method is acid hydrolysis. Chitin can be obtained in several ways. Perhaps the most efficient of these is to go around to the back door of the most prosperous sea food restaurant within easy reach and talk to the official in charge of the garbage cans about a deal for his vacant lobster shells. A slower method is to put shrimp on the family menu as often as necessary and save the peelings. Investigators with a distaste for sea food can get their chitin at this season of the year by putting a corps of small boys to work hunting June bugs (May bugs, that is, in the South).

For those who shun behavior tending to mark them as colorful scientific personalities, there is still another alternative. They can purchase ready-purified D-*Glucosamine Hydrochloride* as Eastman 644. Foreseeing the demand from the shy ones, we have laid in a stock of clean chitin that looks like breakfast food or perhaps fingernail clippings.

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The powers of semi-darkness

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What, then, can be done to encourage and facilitate the generation of lots more slides and filmstrips? Consider the sources.

There are organizations—some for profit, some nonprofit—that produce them for schools. All they need for encouragement is sales. Subjects are of the kind that keep well. Life along the Nile. The circulation of the atmosphere. That sort of thing.

Then there are firms producing films and filmstrips to order for promoters of causes. The need for higher protective tariffs. The need for lower barriers to international trade. How to sell bicycles to people over 40. Here an advertising or public relations agency often acts as intermediary between sponsor and producer.

Outfits that use slides and filmstrips to communicate on a broad and varied scale often maintain their own production facilities for the purpose. This would include large companies, government bureaus, ag colleges.

Not to be neglected beyond these large operations, however, is the individual on his own who has an audience to face and to tell of his work and thoughts as vividly as he can. He, too, can make them—slides if he intends to put on the same performance only once or a few times, filmstrips if it is to be given many times in essentially unchanged form.

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Lecturer in Pharmacology and Toxicology, The Medical Branch, and Professor and Chairman of the Department of Experimental Medicine, The Southwestern Medical School, University of Texas, Dallas

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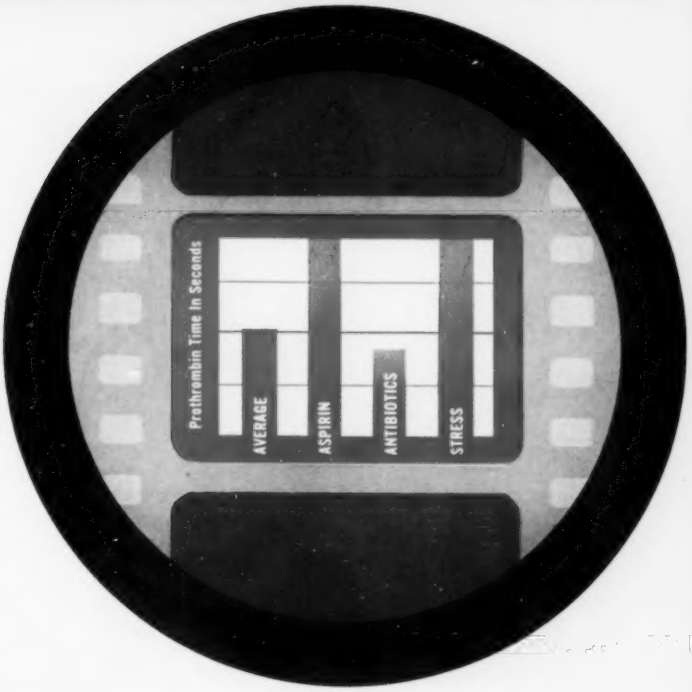

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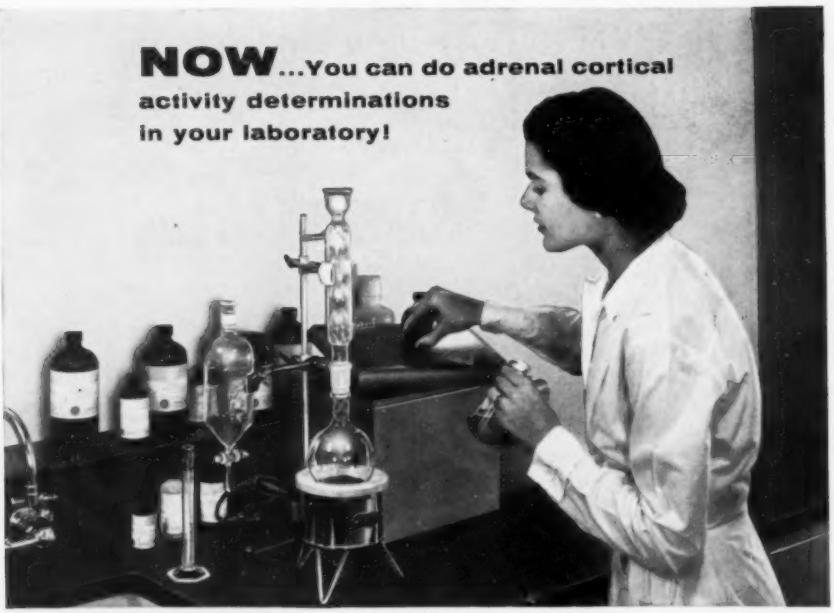

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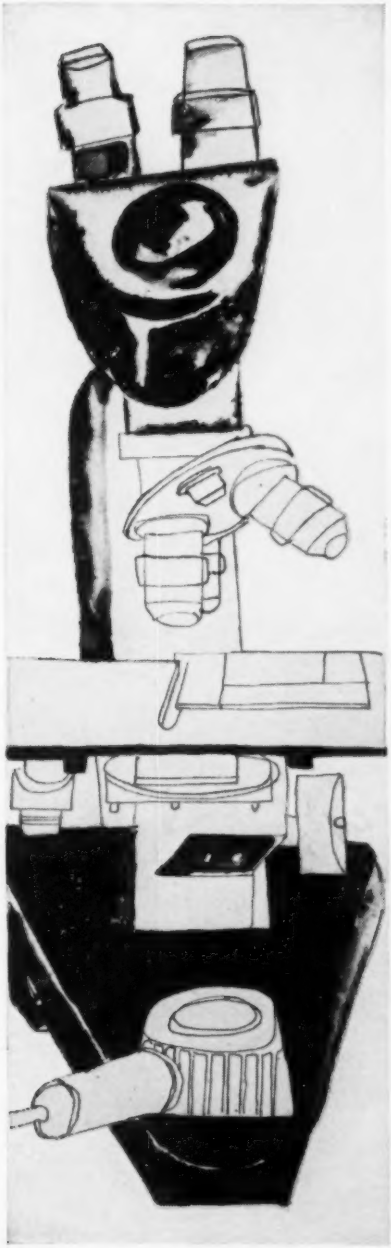

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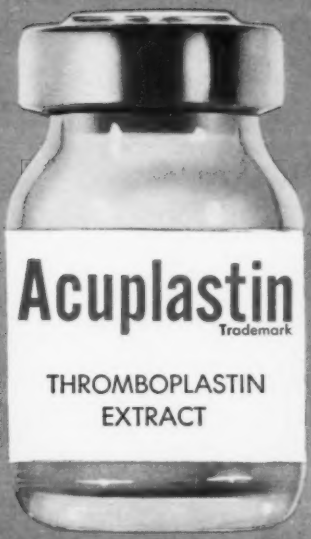

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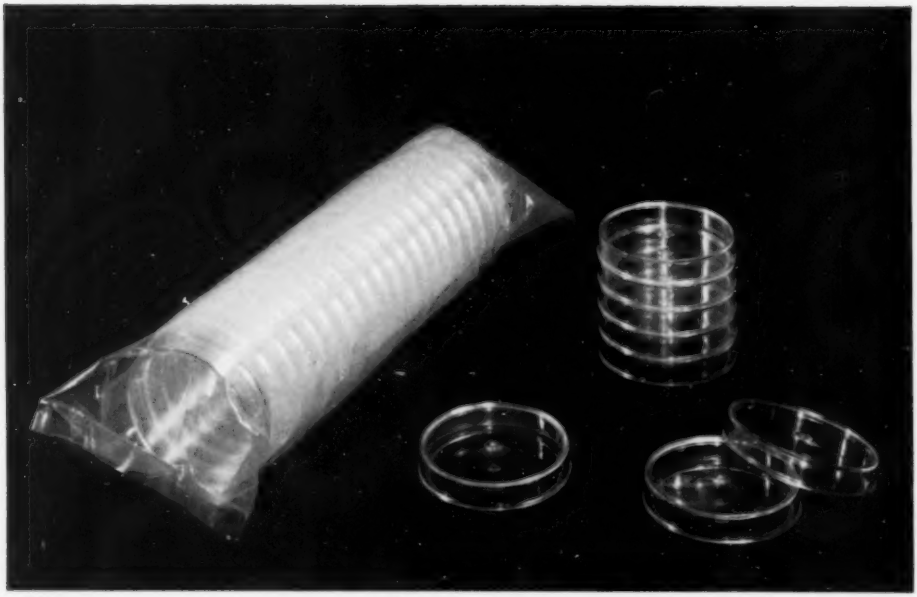

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CLINICAL AND EXPERIMENTAL

THE EFFECT OF PTEROYLGLUTAMIC ACID ADMINISTRATION ON THE SERUM VITAMIN B_{12} CONCENTRATION IN PERNICIOUS ANEMIA IN RELAPSE

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BECAUSE of the effectiveness of parenteral administration of vitamin B_{12} in pernicious anemia, it is generally accepted that the clinical manifestations of this disease are due to a deficiency of this vitamin. This is supported by the low concentration of vitamin B_{12} in the serum observed by Ross[1] and many other workers.[2-4] Factors of the folic acid group also have hemopoietic activity in this disease, but the administration of pteroylglutamic acid (PGA) may provoke the nervous system lesions of vitamin B_{12} deficiency.[5]

The present study deals with the effect of short-term administration of PGA on the serum vitamin B_{12} concentration in untreated Addisonian pernicious anemia.

MATERIALS AND METHODS

Thirteen cases of pernicious anemia have been studied. The diagnosis was based on the generally accepted clinical and laboratory criteria (including serum vitamin B_{12} level). For this study we have selected patients without subjective or objective signs of nervous system disease, because of the potential danger of PGA administration in pernicious anemia.

Patients whose hemoglobin level was less than 6 Gm. per cent were also considered unsuitable, because we did not want to postpone adequate treatment with vitamin B_{12} with this severe degree of anemia.

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With one exception we have studied patients with a serum vitamin B_{12} level higher than 17 $\mu\mu$g per milliliter.

PGA was administered by mouth in a dosage of 15 mg. per day over a period of 5 days in 10 cases, 4 days in 1 case, 6 days in another case, and 8 days in the last case.

The vitamin B_{12} level in the serum was estimated before administration of PGA and on the last day of PGA treatment. The total vitamin B_{12} activity of the serum was determined with the *Lactobacillus leichmannii* A.T.C.C. 7830 Strain 313. Details of the procedure were previously described.[4] Addition of PGA to serum in vitro does not influence the results of the vitamin B_{12} assay.

Seventeen micrograms per milliliter is the lowest level which can be estimated with the method we used. The accuracy of the results is about 15 to 20 per cent in these low ranges of vitamin B_{12} activity. As a criterion for the hemopoietic response we used the reticulocyte count method of Van Herwerden.

The folic acid activity of the serum was determined by using *Streptococcus faecalis* R.[4]

TABLE I. THE EFFECT OF PGA ON THE VITAMIN B_{12} LEVEL AND THE RETICULOCYTE COUNT IN PERNICIOUS ANEMIA IN RELAPSE

CASE NO.	DOSES PGA (15 MG. EACH)	VIT. B_{12} BEFORE PGA	VIT. B_{12} AFTER PGA	DECREASE OF VIT. B_{12} (PER CENT)	RETICULOCYTE RESPONSE (MAX. %)	RESULT
1	5	50	35	30	77	+
2	4	100	75	25	93	+
3	6	50	30	40	107	+
4	5	83	67	19	52	+
5	5	63	39	38	58	+
6	5	92	75	19	56	+
7	5	33	<17	>48	114	+
8	5	88	<17	>81	182	+
9	5	60	30	50	198	+
10	8	83	<17	>79	160	+
11	5	<17	<17	0	151	+
12	5	50	50	0	22*	–
13	5	50	50	0	18*	–

*Subsequent treatment with vitamin B_{12} resulted in a good hemopoietic response.

RESULTS

During the short period of this experiment the administration of PGA did not produce nervous system lesions in pernicious anemia in relapse. Table I gives the data concerning the 13 patients treated with PGA.

The vitamin B_{12} level showed a fall in 10 out of these 13 cases after PGA administration. In one patient the vitamin B_{12} content of the serum was less than 17 $\mu\mu$g per milliliter before PGA was given; therefore, a decrease of the vitamin B_{12} could not be detected in this case. No change of the vitamin B_{12} level was found in 2 patients. It is remarkable that in these 2 cases no reticulocyte crisis was produced by the administration of PGA, while in all the other cases studied by us a significant hemopoietic response was produced by PGA treatment. In these two cases the folic acid activity of the serum was increased during the administration of PGA (Fig. 1).

DISCUSSION

In 10 out of 13 cases of Addisonian pernicious anemia in relapse a decrease of the vitamin B_{12} concentration was associated with a hematologic

response after treatment with PGA. It is striking that the serum vitamin B_{12} value did not change in the 2 patients in whom the administration of PGA did not influence blood production. In one patient who had a reticulocyte response the vitamin B_{12} level both before and after the administration of PGA was lower than the concentration which can be detected with the method used by us. From the data of the last case no conclusions could be drawn.

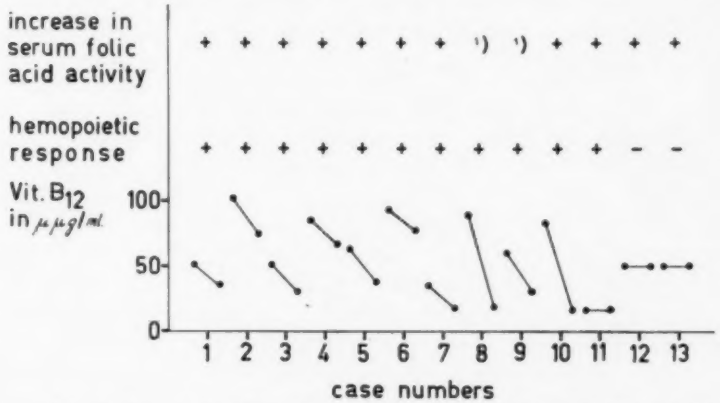

Fig. 1.—Serum vitamin B_{12} level before and after ± 5 days of PGA administration in 13 cases of pernicious anemia in relapse.
1) In these two patients folic acid determinations were not performed.

There seems to exist a relation between the effect of PGA on blood formation and the decrease of the serum vitamin B_{12} level. The significance of our data is indicated by (1) the fact that we never found an increase of the serum vitamin B_{12} level; and (2) the decrease of the vitamin B_{12} level which was more than 20 per cent in 8 out of 10 cases and 19 per cent in the remaining 2 cases.

The conclusion that the decrease of the vitamin B_{12} level is due to the hemopoietic activity of PGA is even more convincing since it could be demonstrated that the extent of the decrease is dependent upon the degree of the hematologic response.

In the graph of Fig. 2 the percentage fall of the serum vitamin B_{12} is plotted against the maximum number of reticulocytes (per thousand erythrocytes) during the reticulocyte crisis after the institution of treatment with PGA. This graph shows a correlation between hemopoietic activity and fall in the vitamin B_{12} level; it indicates that the fall in serum vitamin B_{12} is a function of the hemopoietic activity of PGA. This conclusion has been statistically calculated. The critical relation is $2.18 : 0.37 = 5.7$ and thus $P = 0.001$. In the figure the regression-line is drawn (calculated on $Y = 19 + 2.18X$).*

Other data on the effect of PGA on vitamin B_{12} metabolism are provided by 3 incidental observations of a decrease of the vitamin B_{12} levels when PGA

*We wish to express our thanks to Prof. Dr. S. T. Bok, Director of the "Nederlands Centraal Instituut voor Hersenonderzoek" at Amsterdam, for making the statistical calculation.

was given to cases of megaloblastic anemia of pregnancy due to folic acid deficiency. Serum vitamin B_{12} fell from 1,660 $\mu\mu$g to 600 $\mu\mu$g when PGA was given for one week,[4] from 867 $\mu\mu$g to 150 $\mu\mu$g after 4 days' PGA treatment, and in a third case from 483 $\mu\mu$g to 83 $\mu\mu$g after one day of 15 mg. PGA.[6]

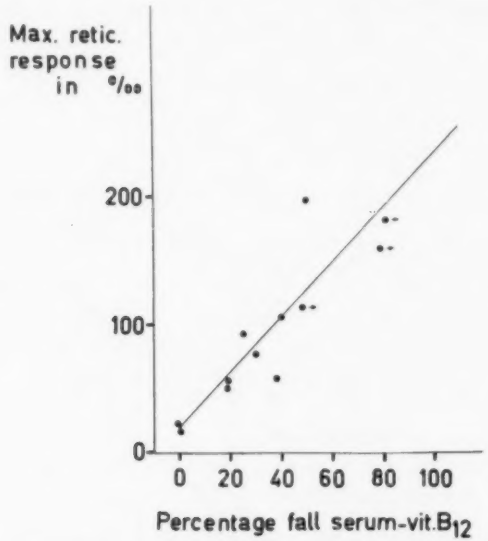

Fig. 2.—Decrease of the serum vitamin B_{12} level (as a percentage of the initial value) as a function of the maximum reticulocyte value (in °/₀₀ of the red blood cell count) after PGA administration. Regression line $Y = 19 + 2.18X$.

Lear and Castle[7] determined the vitamin B_{12} level in patients with pernicious anemia in remission receiving parenteral maintenance treatment of 20 or 30 μg every 2 weeks before and after a period of 6 months of supplemental administration of 5 mg. PGA daily. In 18 out of 27 patients PGA produced a fall of the serum vitamin B_{12} concentration. In this group of patients the hemoglobin values did not change under the influence of PGA nor were signs of nervous system damage seen. A different aspect of the interrelation of folic acid and vitamin B_{12} was observed in a study of the effect of vitamin B_{12} on the blood folic acid activity in pernicious anemia in relapse. A rapid fall of the blood folic acid values occurred, sometimes within 2 hours after the injection.[4] The available data are therefore consistent with the theory that vitamin B_{12} and folic acid act as coenzymes at different stages in the same synthetic process essential for blood formation. This theory was originally proposed by Wright and associates.[8] They suggested that both vitamin B_{12} and folic acid are concerned with the synthesis of nucleic acids. The available data on nucleic acid metabolism in human megaloblastic anemia have been discussed in a previous paper by Nieweg and associates.[4] The present results are consistent with the theory proposed in that report.

It is established by the data obtained that the administration of PGA to patients with pernicious anemia in relapse may produce a change in the distribution of the vitamin B_{12} supply correlated with an increase of blood formation. Presumably more vitamin B_{12} is required by the bone marrow when hemopoiesis is accelerated. It seems plausible that the rapid development of nervous system lesions sometimes associated with PGA administration in vitamin B_{12} deficient subjects is due to a decrease of the amount of vitamin B_{12} available for the nervous system.

SUMMARY

The effect of short-term administration of PGA on the serum vitamin B_{12} level was studied in 13 patients with pernicious anemia in relapse.

In 10 out of 11 cases which showed a hemopoietic response, a decrease of the serum vitamin B_{12} activity could be demonstrated. In the last 2 cases treatment with PGA did not increase blood formation. In these 2 patients the serum vitamin B_{12} level showed no change.

A correlation was established between this decrease of the serum vitamin B_{12} level and the hemopoietic activity of PGA.

These data also suggest that the rapid development of nervous system lesions sometimes associated with PGA administration in vitamin B_{12} deficient subjects is due to a decrease of the amount of vitamin B_{12} available for the nervous system.

REFERENCES

1. Ross, G. I. M.: Vitamin B_{12} Assay in Body Fluids, Nature **166**: 270, 1950.
2. Lear, A. A., Harris, J. W., Castle, W. B., and Fleming, E. M.: The Serum Vitamin B_{12} Concentration in Pernicious Anemia, J. LAB. & CLIN. MED. **44**: 715, 1954.
3. Rosenthal, H. L., and Sarett, H. P.: The Determination of Vitamin B_{12} Activity in Human Serum, J. Biol. Chem. **199**: 433, 1952.
4. Nieweg, H. O., Faber, J. G., de Vries, J. A., and Stenfert Kroese, W. F.: The Relationship of Vitamin B_{12} and Folic Acid in Megaloblastic Anemias, J. LAB. & CLIN. MED. **44**: 1, 118, 1954.
5. Ross, J. F., Belding, H., and Paegel, B. L.: The Development and Progression of Subacute Combined Degeneration of the Spinal Cord in Patients With Pernicious Anemia Treated With Synthetic Pteroylglutamic (Folic) Acid, Blood **3**: 68-90, 1948.
6. Bok, J.: Unpublished data.
7. Lear, A. A., and Castle, W. B.: Supplemental Folic Acid Therapy in Pernicious Anemia: The Effect on Erythropoiesis and Serum Vitamin B_{12} Concentrations in Selected Cases, J. LAB. & CLIN. MED. **47**: 188, 1956.
8. Wright, L. D., Skeggs, H. R., and Huff, J. W.: The Ability of Thymidine to Replace Vitamin B_{12} as a Growth Factor for Certain Lactobacilli, J. Biol. Chem. **175**: 475, 1948.

TREATMENT OF HEPATIC COMA: USE OF CERTAIN KREBS' UREA CYCLE INTERMEDIATES (L-ARGININE, DL-ORNITHINE)

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BOSTON, MASS.

A RELATIONSHIP between disturbances of nitrogen metabolism and the genesis of hepatic coma has been generally accepted and ammonia intoxication may be important in producing this disorder. Although proof is lacking that ammonia is the etiologic agent, the observations that elevated blood ammonia concentrations frequently occur in hepatic coma[1-5] lend support to this thesis. Moreover, impending hepatic coma has been produced in sensitive individuals with liver disease after administration of nitrogenous substances and cation exchange resins[6-10] from which ammonia may be derived.

These considerations have led to treatment of hepatic coma with materials which serve to depress the circulating blood ammonia. Glutamic acid[11, 12] and aspartic acid[13] have been reported to reduce elevated blood ammonia concentrations, although their effect on clinical state and survival has been variable.[14-18]

The recent publication of Najarian and Harper,[19] demonstrating the efficacy of arginine in reducing blood ammonia, stimulated our interest in the use of this and related compounds in treating hepatic coma. This report is an account of our experiences utilizing L-arginine and DL-ornithine both on the general medical and surgical wards and under the more controlled conditions of a metabolic ward.

MATERIALS AND METHODS

Patients.—Ten patients (7 men and 3 women) were treated for hepatic coma, usually with high arterial ammonia concentrations, on the medical and surgical wards of Boston City Hospital, and 5 (4 men, 1 woman) were observed on the Thorndike Metabolic Ward. Clinical data and laboratory findings are tabulated. With one exception (a patient without liver disease) all exhibited unequivocal physical and biochemical evidence of hepatic cirrhosis. In 11, alcoholism was considered important in the genesis of the disease, but the etiology of cirrhosis was unknown in 3 others. Post-mortem examination or surgical biopsy confirmed the diagnosis of cirrhosis in 7. Hepatic coma was thought to be a primary result in hepatic failure (as evidenced by hyperbilirubinemia and the presence of ascites) in 5 instances, thought due to massive gastrointestinal hemorrhage in 2,

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to both gastrointestinal hemorrhage and hepatic failure in 3, to intolerance to dietary nitrogen in 3, to paracentesis in 1, and followed the intravenous administration of massive doses of ammonium chloride in 1 (the patient without liver disease).

Clinical Evaluation of Coma.—Criteria for diagnosis of hepatic coma were those described by Adams and Foley.[20] The following grades were recognized: Grade 1: minimal signs including "flapping" tremor of the hyperextended hands, lethargy, slurred speech, inability to perform simple arithmetic problems, and failure to maintain a neat habitus where this was normally observed, but maintaining orientation and sphincter control. Grade 2: the above findings accentuated, in addition to which patients exhibited disorientation or confusion. Grade 3: a state of stupor from which the patient could be aroused to produce motion or incoherent speech. Grade 4: deep coma. Significant improvement has been defined as recovery of consciousness in an originally stuporous or comatose patient and complete recovery in a patient with impending hepatic coma.

Treatment.—Twenty to fifty grams of L-arginine* was administered intravenously over periods of from 30 minutes to 3 hours. The powder was prepared by dissolving 25 Gm. L-arginine monohydrochloride in 400 ml. sterile, pyrogen-free water, filtering through a Seitz filter, and transferring to sterile containers. DL-Ornithine hydrochloride,† 25 Gm., was similarly prepared. Immediately before infusion, 100 ml. of 50 per cent glucose was added resulting in a 10 per cent glucose solution. In 4 patients infusions were repeated on succeeding days. Arterial blood ammonia concentrations were usually measured twice before the start of treatment and frequently during and after its conclusion.

As indicated in the tables, some patients received additional treatment including protein deprivation, oral carbohydrate alimentation in the form of a partially hydrolyzed starch‡ mixed with fruit juices, enemas and purges, oral nonabsorbable broad spectrum antibiotics (neomycin§ and humycin§), oral and intravenous tetracycline, and hypertonic saline. Gastrointestinal hemorrhage thought to originate from esophageal varices was treated by esophageal tamponage.

Two subjects (J. P. and J. H.) were studied under better controlled conditions on the Thorndike ward. Both received a constant daily 90 Gm. protein diet calculated according to standard tables.[21] J. P., a chronic alcoholic with stabilized portal cirrhosis and hepatosplenomegaly, exhibited persisting intolerance to dietary protein. On a standard 90 Gm. protein intake he developed severe impending hepatic coma which rapidly cleared with administration of an oral nonabsorbable broad spectrum antibiotic in appropriate dosage. On withdrawal of the antibiotic, impending coma recurred in from 7 to 10 days. On 3 occasions, arginine (25 to 50 Gm. daily) was administered intravenously for from 1 to 3 days while the standard protein diet was maintained and in the absence of other forms of therapy (Table III and Figs. 1 and 2).

J. H. had undergone portacaval anastomosis approximately 2 months before studies were begun. Postoperatively she developed episodic stupor responding rapidly to oral nonabsorbable broad spectrum antibiotics. On 2 occasions 25 Gm. of arginine were administered daily for 1 and 3 days in lieu of other treatment. During one day of the second study the development of nausea and vomiting prevented maintenance of the 90 Gm. protein intake.

Three other patients observed on the Thorndike ward had received L-arginine under circumstances similar to those on the general wards.

Laboratory Methods.—A modification[22] of Conway's method[23] was utilized to determine ammonia concentrations in arterial blood. The upper level of normal in this laboratory is 70 μg per cent. Serum bilirubin was determined by the method of Ducci and Watson[24] and blood urea nitrogen by the method of Owings and Mandel.[25]

*Kindly supplied by Dr. Jesse P. Greenstein, National Institutes of Health, Bethesda, Maryland, by Dr. Augustus Gibson, Merck & Company, Inc., Rahway, N. J., as solution, and purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, as a powder.

†Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡Dexin, Burroughs Wellcome & Co., Tuckahoe, N. Y.

§Supplied through the courtesy of Parke, Davis & Co., Detroit, Mich.

TABLE I. CLINICAL AND BIOCHEMICAL DATA OF PATIENTS IN WHOM COMA APPEARED TO BE DUE TO HEPATIC FAILURE ONLY*

PATIENT AGE SEX	TOTAL SERUM BILIRUBIN (MG. %)	DURATION OF COMA	TIME† (− BEFORE) (+ AFTER)	TREATMENT AND AMOUNT	ARTERIAL NH_3 (MG. %)	UREA NITROGEN (MG. %)	GRADE OF HEPATIC COMA	OTHER TREATMENT	COMMENT
J. S.	3.2	24 hr.	−210 min.		91		3	1. Protein deprivation	Complete neuropsychiatric
46			− 30 min.		95	90	3	2. Enema, purge	recovery in 48 hours
M			− 3 min.		81		3	3. Oral tetracycline	
			0	Arginine, 25 Gm.				4. Oral carbohydrate	
			+ 5 min.		71				
			+ 15 min.		57				
			+ 25 min.		56				
			+ 40 min.	Infusion complete	104	90	3		
			+ 24 hr.		47		1		
J. G.	8.8	48 hr.	− 5 min.		70		4	1. Protein deprivation	No improvement. Died
57			0	Arginine, 25 Gm.				2. Enema, purge	
M			+ 2 hr.	Infusion complete	60		4	3. I.V. tetracycline	
			+ 20 hr.		72		4	4. Oral carbohydrate	
E. A.	2.8	24 hr.	−120 min.		73		2		
41			− 30 min.		62		2		
M			0	Arginine, 25 Gm.				None	Slow recovery over many
			+ 15 min.		51		2		weeks
			+ 60 min.	Infusion complete	59		2		
			+ 22 hr.		69		2		

P. L.	16.8	3 days	−210 min.		135		3	1. Protein deprivation	No improvement during arginine treatment and for 48 hours after. Temporary improvement related to other measures. Died in renal failure
54			− 5 min.		168	90	3	2. Enema, purge	
M			0	Arginine, 50 Gm.				3. Oral nonabsorbable broad spectrum antibiotic	
			+ 10 min.		167		3		
			+ 20 min.		141				
			+ 60 min.		95			4. Oral carbohydrate	
			+120 in.		112	84			
			+180 min.	Infusion complete	117		3		
			+ 19 hr.		64				
			+ 3 days		66		1		
S. I.	32	4 days	− 60 min.		171		3	1. Protein deprivation	Slight improvement occurred during 24 to 48 hours after therapy began. Greatly improved after 72 hours. Died in renal failure
60			− 10 min.		169		3	2. Enema, purge	
F			0	Ornithine, 20 Gm.				3. Oral, nonabsorbable broad spectrum antibiotic	
			+120 min.		128				
			+150 min.		111		3		
			+155 min.	Arginine, 25 Gm.					
			+200 min.		80		3		
			+ 4 hr.	Infusion complete	79		3		
			+ 48 hr.		68		2		
			+ 72 hr.				1		

*All patients were alcoholics with cirrhosis and ascites; in 2 (P. L. and S. I.) the diagnosis was confirmed at autopsy.
†Time before and after administration of antibiotic.

TABLE II. CLINICAL AND BIOCHEMICAL DATA OF PATIENTS IN

PATIENT AGE SEX	DIAGNOSIS	PRECIPITATING FACTOR	TOTAL SERUM BILIRUBIN (MG. %)	DURATION OF COMA	TIME† (− BEFORE) (+ AFTER)	TREATMENT AND AMOUNT
R. K. 70 M	Cirrhosis, unknown etiology; esophageal varices; hepatoma (autopsy)	G. I. hemorrhage	3.8	4 days	− 45 min. − 5 min. 0 + 5 min. + 15 min. + 30 min. + 45 min. + 60 min. + 70 min. + 75 min. + 85 min. + 10 hr. + 23 hr. + 24 hr. + 28 hr.	Arginine, 20 Gm. Infusion speed increased Infusion complete Arginine, 25 Gm. (2 hr.) Arginine, 25 Gm. (3 hr.)
J. C. 70 M	Cirrhosis, unknown etiology (biopsy); portacaval anastomosis; episodic stupor	Dietary nitrogen	1.0	16 hr.	− 60 min. − 5 min. 0 + 15 min. + 25 min. + 60 min. + 3 hr. + 6 hr. + 23 hr.	Arginine, 20 Gm. Infusion complete
J. A. 62 M	Portal cirrhosis (alcoholic) (autopsy)	Paracentesis, hyponatremia (serum Na 125 mEq./L.)	2.3	12 hr.	−180 min. − 5 min. 0 + 15 min. + 45 min. + 60 min. + 75 min. +120 min. + 21 hr.	Arginine, 25 Gm. Infusion complete
M. J. 56 M	Duodenal ulcer; pyloric obstruction; metabolic alkalosis; normal liver (laparotomy)	I.V. NH_4Cl 29 Gm. in 20 hours	--	12 hr.	− 90 min. − 60 min. − 1 min. 0 + 25 min. + 40 min. + 60 min. + 19 hr.	Arginine, 25 Gm. Infusion complete
J. K. 39 M	Portal cirrhosis (alcoholic)	G. I. hemorrhage	3.2	12 hr.	−210 min. − 5 min. 0 + 15 min. + 30 min. + 50 min. + 3 hr. + 8 hr. + 24 hr. + 48 hr. + 72 hr. + 76 hr.	Arginine, 25 Gm. Infusion complete Arginine, 25 Gm. Arginine, 25 Gm.

WHOM COMA COULD BE ATTRIBUTED TO A PRECIPITATING FACTOR[a]

ARTERIAL NH_3 (MG. %)	UREA NITROGEN (MG. %)	GRADE OF HEPATIC COMA	OTHER TREATMENT	COMMENT
96		3	1. Tamponage	Thirty minutes after arginine was
115	33	3	2. Protein deprivation	begun and 5 min. after comple-
			3. Enema and purge	tion, hemorrhage recurred. It
117			4. I.V. tetracycline	again recurred 24 hr. later and
91	35	3		was uncontrolled. Patient died
112				
90	33			
81		3		
88		3		
113				
138				
		3		
111		3		
192		4		
189		4	1. Protein deprivation	Complete recovery in 36 hr.
186		4	2. Enema and purge	
			3. Oral nonabsorbable broad	
104			spectrum antibiotic	
67			4. Oral carbohydrate	
93		3		
122		4		
102		4		
68		1		
139		2	1. Protein deprivation	No change in clinical state during
151	47		2. Enema and purge	or after arginine infusion. Dra-
			3. Oral tetracycline	matic clinical response to hyper-
142		2	4. Oral carbohydrate	tonic saline infusion. Patient
122	70	2		died later of sepsis and hepatic
101	47			failure
77		2		
51	51			
127		3	1. Discontinued NH_4Cl infu-	Rapid, complete neurological recov-
154		3	sion	ery. At operation (subtotal gas-
123			2. Gastric suction	trectomy) liver appeared normal.
				Biopsy not obtained
91		3		
64		3		
51		2		
47		2		
126		1	1. Tamponage	Active hemorrhage recurred when
150		1	2. Protein deprivation	infusion completed. Tamponage
			3. Enema and purge	controlled bleeding but bleeding
152		1	4. Oral nonabsorbable broad	recurred. Again controlled and
225			spectrum antibiotic	patient recovered
250		1	5. Oral carbohydrate	
237				
		4		
		4		
		4		
102		2		
		4		

TABLE II

PATIENT AGE SEX	DIAGNOSIS	PRECIPITATING FACTOR	TOTAL SERUM BILIRUBIN (MG. %)	DURATION OF COMA	TIME† (− BEFORE) (+ AFTER)	TREATMENT AND AMOUNT
P. M. 64 M	Portal cirrhosis (alcoholic)	G. I. hemorrhage; hepatic failure	11.6	36 hr.	− 80 min. − 5 min. 0 +100 min. +160 min. +200 min. + 4 hr. + 5 hr.	Ornithine, 25 Gm. Infusion complete
M. M. 65 F	Portal cirrhosis (alcoholic)	G. I. hemorrhage; hepatic failure	10.2	Admitted in coma; no history. At least 2 days	− 30 min. − 10 min. 0 + 5 min. + 12 min. + 16 min. + 20 min. + 30 min. + 60 min. + 48 hr.	Arginine, 20 Gm. Infusion complete
M. O. 45 F	Portal cirrhosis (alcoholic) (autopsy)	Hepatic failure; G. I. hemorrhage	16.4	24 hr.	− 25 min. − 5 min. 0 + 60 min. + 90 min. +120 min. + 20 hr. + 2 days	Arginine, 50 Gm. Infusion complete

*All patients except J. C. and M. J. had ascites.
†Time before and after administration of antibiotic.

RESULTS AND CLINICAL OBSERVATIONS

Patients were divided into 3 groups. Group I (5 patients, Table I) developed hepatic coma in the absence of a recognizable precipitating factor other than severe hepatic disease, whereas in Group II (8 patients, Table II) a causative factor could be identified. This division seemed desirable since the prognosis of those cases with controllable precipitating factors was better than in those in whom control could not be achieved, i.e., hemorrhage and progressive hepatic failure. The 2 patients observed on the Thorndike ward under controlled conditions are considered separately as Group III (Table III).

Earlier experiences[13] indicated that the stage of hepatic coma at which treatment is begun has no measurable influence on prognosis. Duration of coma may be related to prognosis and this information is included in the tables.

The effect of intravenous arginine and ornithine administration on arterial ammonia concentrations and clinical state is shown in the tables. Two Group I subjects (E. A. and J. G.) had normal arterial ammonia concentrations initially and a significant decrease was not observed. J. S. exhibited a slightly elevated ammonia concentration before treatment, and arginine infusion was accompanied by a drop of 25 μg per cent. At the conclusion of the infusion ammonia concentration was higher than the control (104 μg per cent), and the next day a low of 47 μg per cent was observed. The remaining 2 patients in Group I had elevated arterial ammonia levels initially and reductions of 51 μg per cent and

—Cont'd

ARTERIAL NH_3 (MG. %)	UREA NITROGEN (MG. %)	GRADE OF HEPATIC COMA	OTHER TREATMENT	COMMENT
263		4	1. Tamponage	Seizures. Active bleeding persisted.
330			2. Protein deprivation	No improvement. Patient died
			3. Enema and purge	
203		4	4. Oral nonabsorbable broad	
204		4	spectrum antibiotic	
139				
157		4		
117		4	1. Protein deprivation	Seizures. No change. Patient died
119	66	4	2. Cortisone	
			3. Oral tetracycline	
119		4	4. Paraldehyde and dilantin	
90			for seizures	
64			5. Tamponage	
50	65	4		
51		4		
51	67	4		
62		4		
122		3	1. Protein deprivation	Bleeding ceased spontaneously.
125	92	3	2. Enema and purge	Slight (less than 1 grade) improvement during arginine therapy. Later cleared almost completely on "other" treatment. Patient eventually died
			3. Oral nonabsorbable broad	
78		3	spectrum antibiotic	
71		3	4. Oral carbohydrate	
52	95	3		
73		3		
97		4		

91 μg per cent, respectively, were recorded, the latter occurring after successive infusions of ornithine and arginine (S. I.). In no instance did significant improvement in clinical status occur during or immediately after treatment. Three subjects (J. S., S. I., and P. L.) exhibited improvement from 24 to 72 hours after start of therapy, but each was also being treated with a regimen of protein deprivation, oral nonabsorbable broad spectrum antibiotics, and enemas and purges. J. G. showed no clinical change in response to any form of therapy and died within 4 days of admission. The final patient (E. A.) recovered slowly over many weeks coincident with improved liver function. The 3 fatal cases were those with the highest serum bilirubin concentrations.

Elevated arterial ammonia concentrations before therapy were found in every patient in Group II, and all but one exhibited a prompt reduction during the infusion. In one individual (J. K.) a substantial increase of blood ammonia occurred during the infusion of arginine associated with increasing coma due to active, uncontrolled hemorrhage from esophageal varices. This patient received subsequent infusions of 25 Gm. of L-arginine without influence on clinical condition. He began to recover 48 hours after the last infusion when bleeding was finally controlled.

Of 7 patients who exhibited biochemical response to arginine or ornithine infusion, only one showed an improved state of consciousness during or immediately after treatment. Four others later recovered from that episode of

TABLE III. CLINICAL AND BIOCHEMICAL DATA IN PATIENTS WITH HEPATIC COMA PRECIPITATED BY DIETARY NITROGEN STUDIED ON THE METABOLISM WARD*

PATIENT AGE SEX	DIAGNOSIS	TOTAL SERUM BILIRUBIN (MG. %)	DURATION OF COMA	TIME† (− BEFORE) (+ AFTER)	TREATMENT AND AMOUNT	ARTERIAL NH_3 (MG. %)	UREA NITROGEN (MG. %)	GRADE OF HEPATIC COMA	COMMENT
				First Episode					
J. P.	Portal cirrhosis	2.8	12 hr.	− 4 hr.		158		2	Protein intake of 90 Gm. per day maintained. Subsequently, syndrome cleared after oral antibiotic (Fig. 1)
54	(alcoholic);			− 25 min.		115			
M	episodic stupor			− 5 min.		113	18	2	
				0	Arginine, 25 Gm.				
				+ 10 min.		103	11		
				+ 25 min.		65			
				+ 40 min.	Infusion complete	40	18	2	
				+ 60 min.		38		2	
				+ 19 hr.		51		2	
				Second Episode					
			5 days	− 10 min.		114	19	3	Protein intake of 90 Gm. per day maintained. Subsequently, there was further clearing after oral antibiotic (Fig. 1)
				0	Arginine, 25 Gm.				
				+ 30 min.		85			
				+ 60 min.	Infusion complete	74			
				+ 4 hr.	Arginine, 25 Gm. (2 hr.)			3	
				+ 23 hr.		90	19	2	
				+ 24 hr.	Arginine, 25 Gm.				
				+ 27 hr.	Infusion complete	62		2	
				+ 36 hr.	Arginine, 25 Gm. (2 hr.)			2	
				+ 48 hr.		83		2	
				Third Episode					
		1.9	2 days	− 10 min.		145		3	Protein intake of 90 Gm. per day maintained. Slight improvement in 2 days. Subsequently, further clearing after oral antibiotic (Fig. 1)
				0	Arginine, 25 Gm.				
				+ 2 hr.	Infusion complete	92		3	
				+ 24 hr.	Arginine, 25 Gm. (2 hr.)	84		3	
				+ 36 hr.	Arginine, 25 Gm. (2 hr.)			2	

				First Episode					
J. H.	Cirrhosis, unknown	1.1	5 days	− 50 min.		121		2	Protein intake of 90
57	etiology			− 15 min.		119		2	Gm. per day maintained. Great improvement following infusion. After 24 hours recurring mild impending coma (Fig. 2)
F	(biopsy);			0	Arginine, 25 Gm.				
	portacaval anastomosis;			+ 60 min.	Infusion complete	64		2	
	episodic stupor			+ 5 hr.		74		0	
				+ 24 hr.		70		1	
				Second Episode					
			24 hr.	−210 min.		119		3	
				−120 min.		196		3	Nausea and vomiting precluded maintenance of 90 Gm. per day protein intake. (First day, 86 Gm.; second day, 23 Gm.; third day, 75 Gm.) Recurrence of syndrome 4 days after cessation of arginine therapy (Fig. 2)
				− 10 in.		246	19		
				0	Arginine, 25 Gm.				
				+ 3 hr.		151	17	2	
				+ 4 hr.	Infusion complete			2	
				+ 22 hr.	Arginine, 25 Gm.	77	20	1	
				+ 28 hr.	Infusion complete	41	24	1	
				+ 46 hr.	Arginine, 25 Gm.	63		0	
				+ 48 hr.	Infusion complete	51		0	

*No patient in this group had ascites.
†Time before and after administration of antibiotic.

hepatic coma unrelated temporally with arginine therapy. In each case the precipitating factor was eliminated before recovery ensued. In the fatal cases, massive gastrointestinal hemorrhage could not be controlled and one (R. K.) proved to have a hepatoma.

The single Group II example of significant improvement in response to arginine infusion is of special interest since it may represent an unique instance of true "ammoniagenic" coma. A brief case report follows.

M. J., a 56-year-old white man, was admitted after 3 weeks of persistent vomiting due to pyloric obstruction. He had previously imbibed large quantities of whiskey but had abstained for 3 years because of recurring abdominal pain. On admission he was dehydrated, confused, disoriented, lethargic, and exhibited tetany. There were no signs of liver disease. Admission laboratory data revealed hemoconcentration, a profound metabolic alkalosis (CO_2 combining power 70 mEq./L., chloride 46 mEq./L.), and hypokalemia. The urine reaction was acid. Treatment consisted of intravenous fluids and electrolytes including 530 mEq. of ammonium chloride over a 20-hour period. Although serum electrolytes reverted rapidly toward normal in the next 12 hours, the patient's state of consciousness declined and he lapsed into deep coma with extreme muscular rigidity. Despite cessation of ammonium chloride infusion, arterial ammonia concentrations remained elevated for several hours and coma persisted (Table I). Infusion of arginine was associated with mental clearing and complete recovery followed after 16 hours. A typical "flapping" tremor was noted during the period between regaining consciousness and complete recovery. A subtotal gastrectomy was performed later, and at operation the liver appeared to be normal. A biopsy was not obtained.

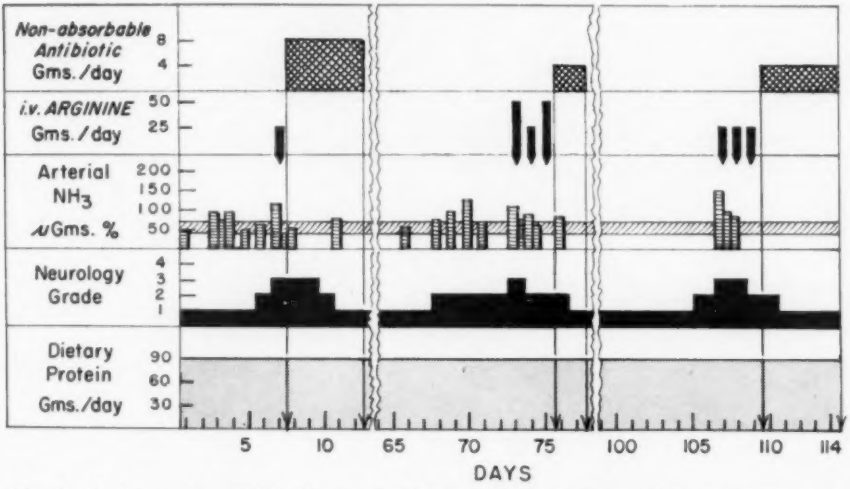

Fig. 1.—Arginine and antibiotic therapy in hepatic coma in J. P., a 54-year-old man with portal cirrhosis and episodic stupor.

Those patients in Group III who developed impending coma as a result of intolerance to dietary nitrogen received infusions of arginine as outlined in Table I and Figs. 1 and 2, during which dietary intake was maintained and no other form of therapy was utilized. J. P. (Fig. 1) failed to respond clinically

to 25 Gm. of arginine although arterial ammonia concentrations diminished. He later cleared when humycin was administered. In a second and third episode he showed slight clinical improvement when arginine was administered for 3 successive days. Again further clearing ensued when the antibiotic was given.

J. H. exhibited prompt, dramatic recovery from severe impending coma within a few hours of administration of 25 Gm. of arginine (Fig. 2). Arterial ammonia concentrations diminished correspondingly. Evidence of impending coma recurred 24 hours later, progressed, and rapidly cleared when humycin was given. A second episode was treated by 3 successive daily infusions of arginine. Again rapid neuropsychiatric clearing ensued, but nausea and vomiting precluded maintenance of the 90 Gm. protein intake. After this course the patient remained free of symptoms for 3 days when recurrence accompanied by high arterial ammonia concentrations developed. Prompt recovery followed antibiotic therapy.

Blood urea nitrogen determinations were obtained during 9 episodes included in this study. In none did a definite and persisting alteration occur following treatment despite significant diminution in ammonia concentrations.

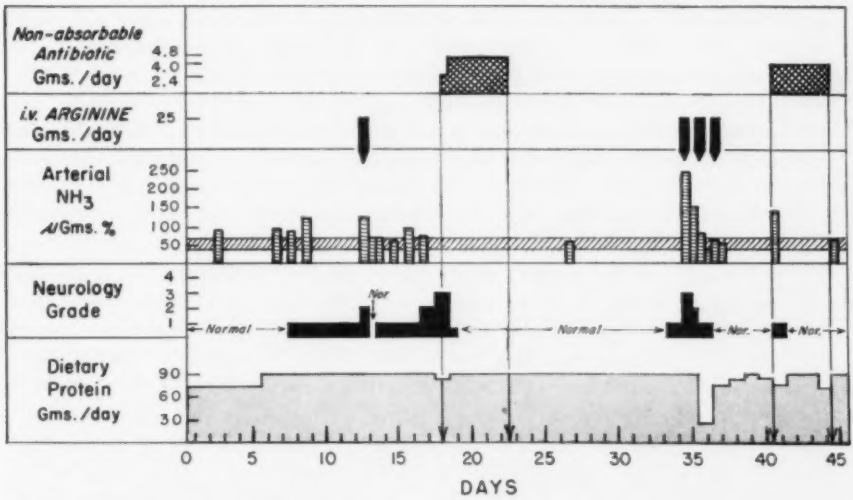

Fig. 2.—Arginine and antibiotic therapy in hepatic coma in J. H., a 57-year-old woman with cirrhosis of unknown etiology, portacaval anastomosis, and episodic stupor.

DISCUSSION

The efficacy of arginine and related compounds in protecting rats against lethal concentrations of amino acid mixtures and ammonium acetate has been clearly demonstrated in a series of observations by Greenstein and coworkers.[26-28] Najarian and Harper[29] extended these studies to dogs, protecting

them against glycine poisoning, thought to be a result to ammonia intoxication, by simultaneous or delayed intravenous administration of arginine, and its protective action was shown to be more effective than that of monosodium glutamate. Later, Najarian and Harper[19] administered arginine to 15 patients with hepatic coma and high blood ammonia concentrations and noted a reduction in ammonia levels, simultaneous elevation of blood urea nitrogen, and at least temporary clinical improvement. Recently Bessman and coworkers[30] administered ammonium chloride intravenously together with sodium glutamate and arginine to patients with and without cirrhosis. Arginine exhibited a slight effect on removal of ammonia from the circulation of the patients with cirrhosis but was inferior to sodium glutamate in this action.

A complete understanding of the basis for the blood ammonia-reducing properties of the compounds under consideration is not presently available. Gullino and associates[26] cited the early studies of Krebs and Hanseleit who observed that the in vitro rate of urea formation from ammonia by liver slices was markedly accelerated by the addition of arginine or ornithine to the reaction. The rise in urea nitrogen observed in protected rats suggested that the major effect of arginine was mobilization of the Krebs-Hanseleit urea-synthesizing cycle. Najarian and Harper similarly observed rising urea nitrogen in both the glycine-poisoned dogs and the human beings with hepatic coma who were treated with arginine and related this to the urea cycle.[19, 29] In a recent Letter to the Editor in *Lancet*, Manning[31] emphasized that it is a fundamental principle of enzymatic reactions that reaction velocity is increased by the addition of increasing amounts of substrate. Therefore, the addition of any of the substrates of the urea cycle would be expected to increase its reaction velocity and hence augment removal of ammonia. He considered arginine the most effective of the urea cycle intermediates due to its free permeability into the liver cell and the high concentration of arginase in liver permitting arginine to act as a precursor to ornithine.

The results of the present study confirm the effectiveness of arginine in reducing blood ammonia concentration. Ornithine appears to act similarly. In contrast to the experience of McDermott and associates[32] ammonia removal began rapidly, usually within a few minutes after the start of the infusion. Evaluation of duration of ammonia reduction was difficult due to utilization of other forms of therapy, although in one of the controlled studies, recurrence of symptoms and rising blood ammonia occurred 24 and 72 hours after cessation of therapy. Corresponding rises in blood urea nitrogen concentrations were not observed—a finding at variance with all previously reported studies and which we cannot explain.

Reliable assessment of a form of therapy is always difficult when employed in treatment of desperately ill patients. In Groups I and II the other measures used were designed to reduce the nitrogen content of the intestines, and included protein deprivation, oral carbohydrate alimentation, enemas and purges, and oral nonabsorbable broad spectrum antibiotics. It has been our experience[13, 34] as well as that of others[35-37] that these are effective means of

reducing blood ammonia concentrations and constitute a treatment program that has increased survival when compared to similar groups of patients treated by other methods.[13] Our results suggest that clinical improvement and ultimate recovery were at least as dependent on those measures as on arginine effect.

Arginine administration with constant protein intake in 2 patients with episodic stupor serves as the best measure of its therapeutic effectiveness. Although in one patient there was an arginine-induced improvement in 2 of 3 impending coma episodes, further clearing was always observed after administration of an oral nonabsorbable broad spectrum antibiotic. The second patient responded rapidly on 2 occasions to arginine therapy. However, equally dramatic and rapid improvement followed antibiotic ingestion. Thus, while arginine possesses excellent blood ammonia-reducing properties, its effectiveness in improving the state of consciousness of patients with hepatic coma and its influence on ultimate survival is not apparently superior to measures designed to reduce absorption of nitrogen from the intestines.

These considerations point up the question of the role of ammonia in the genesis of the hepatic coma syndrome. It is generally accepted that only a very rough correlation exists between venous ammonia concentration and state of consciousness in hepatic disease. Bessman and associates demonstrated a positive arteriovenous ammonia difference which they felt was a result of muscle and cerebral ammonia utilization, and found that the arterial concentration paralleled the clinical state.[38, 39] Summerskill and co-workers[33] confirmed the positive arteriovenous difference in most instances but found poor correlation between clinical state and arterial concentrations. They demonstrated further that the introduction of the regimen designed to reduce intestinal nitrogen absorption resulted in lowering of arterial ammonia concentration despite persistence of hepatic coma, indicating that ammonia concentrations reflect the amount of nitrogen absorption from the intestine at the time the blood is collected, rather than clinical state.

There is ample evidence, however, indicating that ammonia toxicity has some role in the production of hepatic coma. Administration of ammonium salts[6, 7, 9] and ammonia-liberating cation exchange resins[8] undoubtedly produce hepatic coma in sensitive patients with cirrhosis. Development of the typical syndrome in man by giving ammonium salts in the absence of severe liver disease has not been thought possible[40] but the case reported here of a patient with normal liver function developing coma as a result of massive intravenous ammonium chloride infusion may refute that thesis. Stauffer[41] has made similar observations in another example of ammonium chloride therapy for metabolic alkalosis in the absence of liver disease. Jaquez and associates[42] found correlation between clinical state and partial pressure of ammonia (PNH_3) closer than between total blood ammonium (NH_3-NH_4^+) and depth of coma. Both Stauffer's patient and ours exhibited severe alkalosis, and as elevation of pH increases the ammonia fraction (PNH_3) it is possible that development of the syndrome in otherwise normal individuals required a combination of both high ammonia concentration and the presence of alkalosis.

Observations that clinical improvement, when it occurs, lags behind return to normal arterial ammonia concentrations suggest either that other factors are important in maintaining the neurological state or that the effect of ammonia on cerebral metabolism is only slowly reversible. The two controlled studies indicated that signs of coma could be rapidly eliminated with appropriate therapy. Of interest was the further improvement in one patient when an antibiotic was given after ammonia concentrations had been reduced by several days of arginine treatment, suggesting that the antibiotic may reduce intestinal formation of toxic substances other than ammonia which could reach the brain by passing unmetabolized through a diseased liver or around it via the collateral circulation.[43] The identification of such material has not been established, although several have been suggested. Methionine may produce impending coma in sensitive subjects with hepatic disease without constant elevation of blood ammonia,[44] and products of its breakdown—methionine sulfoxide, dimethyl sulfide, and methyl mercaptan—have been implicated as possibly being important in production of the syndrome.[45, 46] Oral chlortetracycline prevents this methionine effect. Indoles of intestinal origin may be responsible for some of the manifestations of coma,[39] and while broad spectrum antibiotics could reduce their production by inhibiting bacterial decomposition of protein, arginine, or any related compound would have no effect. Thus a regimen designed both to reduce the amount of intestinal nitrogen, and the bacterial decomposition of that which cannot be removed, i.e., urea, appears to be most successful in protecting the brain from ammonia or any other toxin of intestinal origin.

Ultimate recovery clearly depended on control of factors precipitating coma. The Group I survivors exhibited the least derangement of liver function (serum bilirubin < 5 mg. per cent) and the patient surviving after massive gastrointestinal hemorrhage improved only in relation to control of bleeding. Indeed, during active bleeding intestinal nitrogen absorption was so great[47] that ammonia concentrations increased during infusion of arginine. The patient with pure ammoniagenic coma would probably have recovered without therapy, and there is ample evidence that episodic stupor responds to dietary protein reduction. The final survivor exhibited no improvement until hypertonic saline was given to correct the paracentesis-induced disorder leading to coma.

Systematic review of Najarian and Harper's case reports[19] reveals results essentially similar to ours. Improvement and recovery in all cases of coma induced by gastrointestinal hemorrhage and dietary nitrogen were clearly related to removal or control of precipitating factors. Four episodes of coma complicating infectious hepatitis were reported. In one, clinical improvement could be associated only with arginine therapy, while in another insufficient data as to ancillary treatment and diet were provided. A third patient failed to respond, and in the fourth mental clearing after arginine therapy was coincident with rapid improvement in liver function; unless one ascribes to arginine some effect on the course of viral hepatitis, its administration was

not clearly related to the outcome. A patient with hepatic lymphosarcoma exhibited a favorable response after arginine infusion but details of other treatment and diet were not provided.

Glutamic acid, sodium glutamate, and aspartic acid have also exhibited blood ammonia-reducing properties but clinical results have been variable and their interpretation rendered difficult by omission of details of other features of the patients' care. It should be emphasized that evaluation of any specific therapy for hepatic coma must be conducted with control and clear exhibition of the amount of dietary nitrogen, employment of antibiotics, and the nature of precipitating factors.

In another report[34] the results of treatment with humycin and neomycin are presented. Under controlled conditions clinical response to these agents was rapidly favorable and administration uncomplicated, in contrast to the difficulties of intravenous therapy. The clinical use of intravenously administered agents to reduce blood ammonia appears to have only limited application in treatment of hepatic coma.

SUMMARY

L-Arginine and DL-ornithine have been administered to 15 subjects with various degrees of hepatic coma and observations made of their effect on arterial ammonia concentrations and clinical state.

In the majority, a prompt reduction of arterial ammonia concentration occurred with, or immediately following, the infusion. Clinical response usually could not be related to the reduction in blood ammonia and seemed to be more closely associated with the control of factors precipitating coma and to measures designed to reduce intestinal nitrogen absorption. Two subjects receiving arginine while on a controlled, constant protein intake exhibited clinical improvement which was neither more rapid nor more complete than that achieved by administration of oral nonabsorbable broad spectrum antibiotics.

It is concluded that arginine, ornithine, and other agents designed solely to reduce ammonia concentration by intravenous infusion have limited value in treatment of hepatic coma.

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REFERENCES

1. Van Caulaert, C., Deviller, C., and Halff, M.: Le taux de l'ammoniémie dans certaines affections hépatiques, Compt. rend. Soc. biol. 111: 735, 1932.
2. Schwartz, R., Phillips, G. B., Gabuzda, G. J., Jr., and Davidson, C. S.: Blood Ammonia and Electrolytes in Hepatic Coma, J. Lab. & Clin. Med. 42: 499, 1953.
3. Seegmiller, J. E., Schwartz, R., and Davidson, C. S.: The Plasma "Ammonia" and Glutamine Content in Patients With Hepatic Coma, J. Clin. Invest. 33: 984, 1954.
4. Traeger, H. S., Gabuzda, G. J., Jr., Ballou, A. N., and Davidson, C. S.: Blood "Ammonia" Concentration in Liver Disease and Liver Coma, Metabolism 3: 99, 1954.
5. Phear, E. A., Sherlock, S., and Summerskill, W. H. J.: Blood-Ammonium Levels in Liver Disease and "Hepatic Coma," Lancet 1: 836, 1955.

6. Van Caulaert, C., and Deviller, C.: Ammoniémie expérimentale après ingestion de chlorure d'ammonium chez l'homme à l'état normal et pathologique, Compt. rend. Soc. biol. **111**: 50, 1932.
7. Kirk, E.: Amino Acid and Ammonia Metabolism in Liver Diseases, Acta med. scandinav. (Suppl.) **77**: 1, 1936.
8. Gabuzda, G. J., Jr., Phillips, G. B., and Davidson, C. S.: Reversible Toxic Manifestations in Patients With Cirrhosis of the Liver Given Cation-Exchange Resins, New England J. Med. **246**: 124, 1952.
9. Phillips, G. B., Schwartz, R., Gabuzda, G. J., Jr., and Davidson, C. S.: The Syndrome of Impending Hepatic Coma in Patients With Cirrhosis of the Liver Given Certain Nitrogenous Substances, New England J. Med. **247**: 239, 1952.
10. Schwartz, R., Phillips, G. B., Seegmiller, J. E., Gabuzda, G. J., Jr., and Davidson, C. S.: Dietary Protein in the Genesis of Hepatic Coma, New England J. Med. **251**: 685, 1954.
11. Walshe, J. M.: The Effect of Glutamic Acid on the Coma of Hepatic Failure, Lancet **1**: 1075, 1953.
12. Walshe, J. M.: Glutamic Acid in Hepatic Coma, Lancet **1**: 1235, 1955.
13. Summerskill, W. H. J., Wolfe, S. J., and Davidson, C. S.: The Management of Hepatic Coma in Relation to Protein Withdrawal and Certain Specific Measures, Am. J. Med. **23**: 59, 1957.
14. Riddell, A. G., and McDermott, W. V.: Hepatic Coma, Lancet **1**: 1263, 1954.
15. Sherlock, S., Summerskill, W. H. J., White, L. P., and Phear, E. A.: Portal-Systemic Encephalopathy, Lancet **2**: 453, 1954.
16. Singh, I. D., Barclay, J. A., and Cooke, W. T.: Blood NH_3 Levels in Relation to Hepatic Coma and the Administration of Glutamic Acid, Lancet **1**: 1004, 1954.
17. Webster, L. T., Jr., and Davidson, C. S.: The Effect of Sodium Glutamate on Hepatic Coma, J. Clin. Invest. **35**: 191, 1956.
18. Schwartz, I. R., Lehman, E., Hammond, J., Seibel, J. M., and Goldson, F.: The Failure of Monosodium Glutamate in the Treatment of Hepatic Coma, Gastroenterology **30**: 869, 1956.
19. Najarian, J. S., and Harper, H. A.: A Clinical Study of the Effect of Arginine on Blood Ammonia, Am. J. Med. **21**: 832, 1956.
20. Adams, R. D., and Foley, J. M.: The Neurological Disorder Associated With Liver Disease. Research Publications of the Association for Research in Nervous and Mental Diseases, Baltimore, 1953, The Williams & Wilkins Co.
21. Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared, U. S. Dept. Agriculture Handbook No. 8, Washington, D. C., 1950.
22. McDermott, W. V., and Adams, R. D.: Episodic Stupor Associated With an Eck Fistula in the Human With Particular Reference to the Metabolism of Ammonia, J. Clin. Invest. **33**: 1, 1954.
23. Conway, E. J.: Microdiffusion Analysis and Volumetric Error, ed. 3, London, 1951, C. Crosby Lockwood & Son, Ltd.
24. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt-Reacting and the Chloroform-Soluble Types, J. Lab. & Clin. Med. **30**: 293, 1945.
25. Owings, R. H., and Mandel, E. E.: Studies in Non-protein Nitrogen. I. A Convenient Method for Measuring Urea in Blood, Proc. Soc. Exper. Biol. & Med. **78**: 363, 1951.
26. Gullino, P., Winitz, M., Birnbaum, S. M., Cornfield, J., Otey, M. C., and Greenstein, J. P.: Studies on the Metabolism of Amino Acids and Related Compounds in Vivo. I. Toxicity of Essential Amino Acids, Individually and in Mixtures, and the Protective Effect of L-Arginine, Arch. Biochem. **64**: 319, 1956.
27. Greenstein, J. P., Winitz, M., Gullino, P., Birnbaum, S. M., and Otey, M. C.: Studies on the Metabolism of Amino Acids and Related Compounds in Vivo. III. Prevention of Ammonia Toxicity by Arginine and Related Compounds, Arch. Biochem. **64**: 342, 1956.
28. du Ruisseau, J. P., Greenstein, J. P., Winitz, M., and Birnbaum, S. M.: Studies on the Metabolism of Amino Acids and Related Compounds in Vivo. IV. Blood Ammonia and Urea Levels Following Intraperitoneal Administration of Amino Acids and Ammonium Acetate, and the Effect of Arginine Thereon, Arch. Biochem. **64**: 355, 1956.
29. Najarian, J. S., and Harper, H. A.: Comparative Effect of Arginine and Monosodium Glutamate on Blood Ammonia, Proc. Soc. Exper. Biol. & Med. **92**: 560, 1956.
30. Bessman, S. P., Shear, S., and Fitzgerald, J.: Effect of Arginine and Glutamate on the Removal of Ammonia From the Blood in Normal and Cirrhotic Patients, New England J. Med. **256**: 941, 1957.
31. Manning, R. T.: Letter to the Editor, Lancet **1**: 937, 1957.

32. McDermott, W. V., Jr., Henneman, D. H., and Laumont, C.: The Metabolic Effects of Glutamic Acid and Arginine in "Hepatic Coma" With Hyperammoniemia, J. Clin. Invest. **36**: 913, 1957.
33. Summerskill, W. H. J., Wolfe, S. J., and Davidson, C. S.: The Metabolism of Ammonia and α-Keto-Acids in Liver Disease and Hepatic Coma, J. Clin. Invest. **36**: 361, 1957.
34. Fast, B. B., Wolfe, S. J., Stormont, J. M., and Davidson, C. S.: Antibiotic Therapy in the Management of Hepatic Coma, Arch. Int. Med. **101**: 467, 1958.
35. Sherlock, S., Summerskill, W. H. J., and Dawson, A. M.: The Treatment and Prognosis of Hepatic Coma, Lancet **2**: 689, 1956.
36. Fisher, C. J., and Faloon, W. W.: Episodic Stupor Following Portacaval Shunt. Observations on Etiology and Therapy, New England J. Med. **255**: 589, 1956.
37. Fisher, C. J., and Faloon, W. W.: Blood Ammonia Levels in Hepatic Cirrhosis. Their Control by the Oral Administration of Neomycin, New England J. Med. **256**: 1030, 1957.
38. Bessman, S. P., and Bessman, A. N.: The Cerebral and Peripheral Uptake of Ammonia in Liver Disease With an Hypothesis for the Mechanism of Hepatic Coma, J. Clin. Invest. **34**: 622, 1955.
39. Bessman, S. P., and Bradley, J. E.: Uptake of Ammonia by Muscle; Its Implications in Ammoniagenic Coma, New England J. Med. **253**: 1143, 1955.
40. Walshe, J. M.: Hepatic Coma, Postgrad. Med. J. **32**: 467, 1956.
41. Stauffer, J.: Personal communication.
42. Jaquez, J. A., Poppell, J. W., Lawrence, W., Jr., and Roberts, K. E.: Clinical Significance of the Partial Pressure of Ammonia (PNH_3) in Patients With Ammonium Toxicity, Clin. Res. Proc. **5**: 20, 1957.
43. White, L. P., Phear, E. A., Summerskill, W. H. J., and Sherlock, S.: Ammonium Tolerance in Liver Disease: Observations Based on Catheterization of the Hepatic Veins, J. Clin. Invest. **34**: 158, 1955.
44. Phear, E. A., Ruebner, B., Sherlock, S., and Summerskill, W. H. J.: Methionine Toxicity in Liver Disease: Protective Action of Chlortetracycline, Clin. Sc. **15**: 93, 1956.
45. Walshe, J. M.: Observations on the Symptomatology and Pathogenesis of Hepatic Coma, Quart. J. Med. (N.S.) **20**: 421, 1951.
46. Challenger, F., and Walshe, J. M.: Methyl Mercaptan in Relation to Foetor Hepaticus, Biochem. J. **59**: 372, 1955.
47. Young, P. C., Burnside, C. R., Knowles, H. C., Jr., and Schiff, L.: The Effect of Intragastric Administration of Whole Blood on the Concentration of Blood Ammonia in Patients With Liver Disease, J. Lab. & Clin. Med. **50**: 11, 1957.

STUDIES OF NEUROHYPOPHYSEAL FUNCTION IN MAN: EFFECT OF ADRENAL STEROIDS ON POLYURIA IN COMBINED ANTERIOR AND POSTERIOR PITUITARY INSUFFICIENCY

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AMELIORATION of polyuria in posterior pituitary insufficiency by destruction of the anterior lobe of the pituitary gland[1] has been attributed by some workers to removal of various diuretic anterior pituitary hormones.[2-4] However, Leaf and associates[5] have suggested that these hormones are not diuretic in themselves but influence water metabolism indirectly through their effects on solute intake and excretion. The present report describes studies of a patient with post-partum pituitary necrosis (Sheehan's syndrome) and combined anterior and posterior pituitary insufficiency in whom a diuretic action of adrenal steroids has been demonstrated which may be tentatively attributed to an inhibitory effect of these hormones on secretion of antidiuretic hormone (ADH) from the hypothalamus.† A second patient with a similar disorder who demonstrated polyuria only with administration of adrenal steroids is also briefly reported.

MATERIALS AND METHODS

The technique of study of neurohypophyseal function with intravenously administered nicotine, hypertonic saline, and Pitressin has been previously described.[7] Acute changes in urinary total solute concentration (Uosm) (measured with a Fiske osmometer) and in the renal excretion of solute-free water (CH_2O)[8] were measured as indices of ADH secretion. Other measurements included urinary total 17-hydroxycorticosteroid (17-OHCS),[9] 17-ketosteroid (17-KS),[10] follicle stimulating hormone (FSH) assays,[11] serum protein bound iodine (P.B.I.),[12] and serum and urinary concentrations of creatinine (Cr),[13] chloride (Cl),[14] sodium, and potassium (using a Baird flame photometer with lithium as the internal standard).

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RESULTS

CASE 1.—M. B. (PBBH 7E 760), a 26-year-old housewife, developed afibrinogenemia, profuse uterine hemorrhage, and acute renal failure during delivery of her second child on Nov. 25, 1952. The patient subsequently underwent artificial hemodialysis, following which she gradually improved. Prior to discharge, urine volume was approximately 1.0 L. per day, blood urea nitrogen was 19 mg. per 100 ml., and phenosulfonphthalein excretion 25 per cent in 20 minutes. The patient was next seen in July, 1953, complaining of polydipsia, polyuria, amenorrhea, loss of axillary and pubic hair, loss of libido, fatigue, and cold intolerance. Laboratory studies were suggestive of gonadotrophic and corticotrophic insufficiency but thyroid function was normal (Table I).

TABLE I. LABORATORY DATA IN PATIENT M. B.

DATE	TREATMENT*	24-HR. URINE VOL. (L.)	U_{osm} (mOsm./L.)	17-OHCS (MG.)	17-KS (MG.)	B.M.R. (%)	I^{131} (%)	P.B.I. (μG./%)	F.S.H. (MOUSE UNITS)	B.W. (POUNDS)
7/21/53	None	3.4				+13	28		< 10	120
7/24/53	None	2.8		0.0	4.9					
7/25/53	ACTH†	2.7		1.1	6.9					
7/26/53	ACTH	3.7		8.4	11.8					
7/27/53	ACTH	3.0		–‡	13.2					
3/17/54	None	2.5	215	0.0	4.3	–25	6	2.4	< 10	132
5/ 9/54	T 60	1.8§	264							
5/10/54	T 60	0.9‖	315	1.8	2.4					120
5/25/54	E 25									
	T 90	1.7‖	207			–25				
6/ 9/54	E 25									
	T 90	2.9	228			+ 1				122
10/ 5/54	E 37.5									
	T 90	2.7	195							
3/ 9/55	E 37.5									
	T 90	3.5	181							135
5/26/55	E none¶									
	T 90	2.3	176			–18				
5/27/55	E none**									
	T 90	2.6	197							
9/ 3/55	E 25									
	T 120	3.3	183							
12/ 7/55	E 50									
	T 120	5.2	167							
1/ 6/56	E 25									
	T 120	2.9	224							
5/15/56	E 25									
	T 120	2.7	168							143

*E = cortisone; T = desiccated thyroid. The patient received 0.5 mg. stilbestrol daily after 3/17/54.
†Twenty-five milligrams ACTH intravenously for 8 hours daily.
‡Not measured.
§Sixteen-hour collection.
‖Fourteen-hour collection.
¶Off cortisone for 24 hours.
**Off cortisone for 48 hours.

The patient was instructed to take 25 mg. cortisone by mouth daily, but which she took only intermittently in the ensuing months, and classic symptoms and signs of myxedema were present when she was next examined in March, 1954 (Table I). The patient was treated daily with thyroid and estrogenic hormones but the B.M.R. remained depressed until cortisone therapy was begun in May, 1954, following which it rose to normal.

Studies of neurohypophyseal function were performed in this patient prior to and during replacement therapy with estrogenic, thyroid, and adrenal

steroid hormones (Table II). In July, 1953, there was a normal antidiuretic response to small intravenous doses of nicotine and Pitressin but essentially no response to intravenous hypertonic saline (Table II, *A*). With the development of myxedema, there was slight delay in reaching peak water diuresis, which was somewhat depressed, and the antidiuretic response to 1.0 mg. nicotine was profound and prolonged (Table II, *B*). Hypertonic saline was infused slowly for a period to provide electrolyte for adequate renal excretion of water,[15] and then administered rapidly, but no antidiuretic response ensued.

Therapy with estrogenic and thyroid hormones did not improve the delayed and depressed water diuresis and a prolonged antidiuresis was again

TABLE II. STUDIES OF NEUROPHYSEAL FUNCTION IN PATIENT M. B.

A. (7/23/53) Hypogonad, Hypoadrenal, and Euthyroid

TIME (MIN.)	TREATMENT	V (ML./MIN.)	U_{CL} (mEq./L.)	P_{CL} (mEq./L.)	U_{CR} (MG. %)	$U_{CR}V$ (MG./MIN.)
0	20 ml. water/Kg. p.o.*	1.8†	12.4	107	40.9	0.7
45- 60		6.2	13.1		11.9	0.7
75	Nic. 1.0 mg. I.V.					
105-135		1.3	23.1			0.5
195-210		4.3	7.1	104	16.7	0.7
210-270	Pit. 15 mu. I.V.	0.8	–		79.5	0.6
360-390		4.5	21.0	102	13.7	0.6
390-428	3% saline I.V. 500 ml.	2.7‡	41.9	113	15.8	0.4

B. (3/18/54) Hypogonad, Hypoadrenal, and Hypothyroid

TIME (MIN.)	TREATMENT	V (ML./MIN.)	U_{OSM} (MOSM./L.)	P_{OSM} (MOSM./L.)	C_{OSM} (ML./MIN.)	C_{H_2O} (ML./MIN.)	C_{CR} (ML./MIN.)
0	20 ml. water/Kg. p.o.*	0.8†	457	289	1.3	–0.5	49
90-105		4.7	139	276	2.3	+2.3	64
110	Nic. 1.0 mg. I.V.						
150-180		0.8	363	279	1.1	–0.3	34
195-225		1.3	454		2.2	–0.8	76
325	3% saline I.V. 1.0 ml./min.						
345-375		2.2	309	274	2.4	–0.3	78
435	3% saline IV. 2.0 ml./min.						
450-465		4.0	259	286	3.7	+0.3	63
470-492	3% saline I.V. 500 ml.	7.5‡	223	294	5.7	+1.8	44

C. (5/11/54) Hypoadrenal; 60 mg. Thyroid and 0.5 Stilbestrol Daily

		0.5§	262				
0	20 ml. water/Kg. p.o.*	0.2†	447	299	0.3	–0.1	42
120-135		5.7	180	285	3.6	+2.1	91
135	Nic. 0.5 mg. I.V.						
195-225		1.6	278	277	1.6	0.0	79
270	3% saline I.V. 1.0 ml./min.						
330	3.0 ml./min.						
375-405		2.2	289		2.2	0.0	96
411-433	3% saline 500 ml. I.V.	7.4‡	227	294	5.7	+1.7	94
460	Pit. 15 mu. I.V.						
480-500		2.4	447		3.6	–1.2	83

TABLE II—CONT'D

TIME (MIN.)	TREATMENT	V (ML./MIN.)	U_{OSM} (MOSM./L.)	P_{OSM} (MOSM./L.)	C_{OSM} (ML./MIN.)	C_{H_2O} (ML./MIN.)	C_{CR} (ML./MIN.)
	D. (6/10/54) 25 mg. Cortisone, 90 mg. Thyroid, and 0.5 mg. Stilbestrol Daily						
		1.7§	38				
−150	Cortisone 50 mg. p.o.						
0	20 ml. water/Kg. p.o.*	2.9†	190	289	1.9	+1.0	86
75- 90		13.0	93	277	4.4	+8.6	109
125	Nic. 0.5 mg. I.V.						
165-195		10.6	107	278	4.1	+6.5	87
195	Pit. 15 mu. I.V.						
210-225		2.3	419		3.5	−1.2	79
285-300		9.2	109	272	3.7	+5.5	87
306	Nic. 0.5 mg. I.V.						
360-375		5.5	121	267	2.5	+3.0	75
437-471	3% saline 540 ml. I.V.	6.7‡	122	289	2.8	+3.8	77
	E. (5/28/55) 3 Days off Cortisone; 90 mg. Thyroid, and 0.5 mg. Stilbestrol Daily						
		2.0§	181				
0	20 ml. water/Kg. p.o.*	1.0†	276	287	0.9	+0.04	35
60 -75		5.7	96		1.9	+3.8	48
75	Nic. 0.5 mg. I.V.						
105-120		8.5	83	280	2.5	+6.0	71
135	Nic. 1.0 mg. I.V.						
165-180		1.7	304	271	1.9	−0.2	52
240-255		4.7	120		2.1	+2.6	56
260-300	3% saline 630 ml. I.V.	7.3‡	231	297	5.7	+1.6	59
330	Pit. 15 mu. I.V.						
360-375		3.5	382		4.6	−1.1	67
405-495	Hydrocortisone 100 mg. I.V., 800 ml. water p.o.						
570-585		10.3	84	274	3.1	+7.2	66
585	Nic. 1.0 mg. I.V.						
615-630		5.3	152		2.9	+2.4	88

mu. = milliunits.

*Hydration maintained by administration of water orally or as 5 per cent dextrose in water intravenously at a rate equal to urine flow. Water administration was discontinued prior to saline infusion to decrease the fluid balance to approximately 10 ml./KG. in order to minimize dilution of the hypertonic saline in the extracellular fluid compartment.

†Minimum morning urine flow following overnight dehydration.

‡Minimum urine flow during or after 3 per cent saline infusion.

§Overnight urine (10 P.M. to 8 A.M.).

observed with 0.5 mg. nicotine (Table II, *C*). There was no antidiuretic response to hypertonic saline but a normal response to Pitressin was readily apparent suggesting that the renal tubular action of ADH had not been modified by the associated solute diuresis.[16]

Another study of neurohypophyseal function was performed one month after institution of cortisone therapy (Table II, *D*). Fifty milligrams of cortisone were administered orally 150 minutes prior to hydration and urinary osmolarity decreased with this treatment despite continued dehydration. A normal diuresis of solute-free water occurred with hydration and 0.5 mg. nicotine administered on two separate occasions elicited essentially no antidiuretic response. There was no antidiuresis following hypertonic saline infusion but the effect of Pitressin was not altered by cortisone therapy.

The patient was treated with 0.5 mg. stilbestrol, 90 to 120 mg. desiccated thyroid, and 25 to 50 mg. cortisone daily thereafter. Cortisone was withheld for a 3-day period in May, 1955, and another study of neurohypophyseal function was performed (Table II, *E*). Dilution of the urine with hydration was as great as in the preceding study with cortisone, but the diuresis of solute-free water was moderately depressed. There was no antidiuretic response to 0.5 mg. nicotine, but 1.0 mg. produced a substantial antidiuresis which was

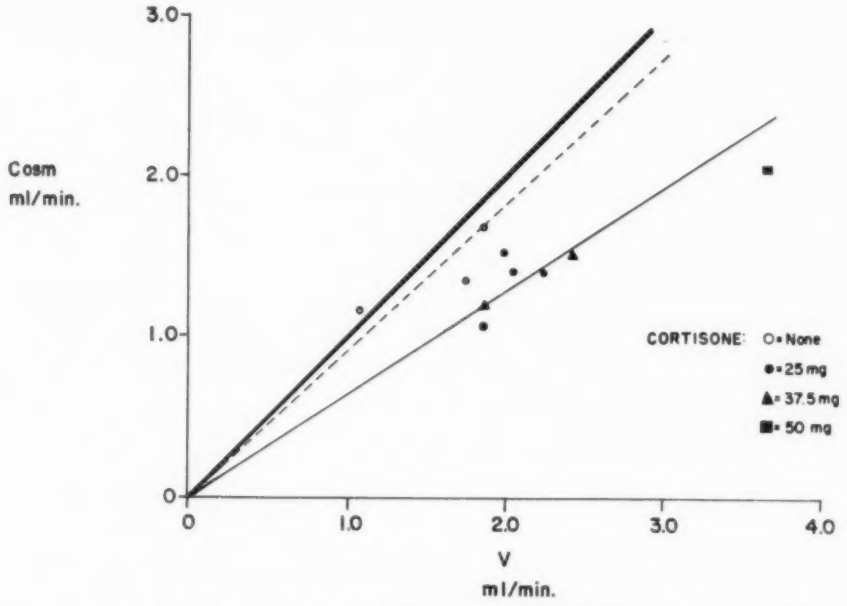

Fig. 1.—Effect of cortisone on urine flow, C_{osm} and C_{H_2O} in Patient M. B. The rates per minute of urine flow (V) and osmolar clearance (C_{osm}) were calculated from specimens listed in Table I collected over 14- to 24-hour periods on ad libitum dietary and fluid intakes. The heavy isobar from the origin represents isosmotic urine excretion. The dashed line represents the mean osmolar U/P ratio for 3 specimens obtained prior to cortisone therapy; the light solid line represents the mean value for 7 specimens obtained on various doses of cortisone. The rate of solute-free water clearance (C_{H_2O}) in ml./min. at various rates of C_{osm} is represented by the horizontal distance of each point from the isosmotic line. Points to the right and left of the line represent positive and negative C_{H_2O}, respectively. Mean values before cortisone were C_{osm} 1.40 ml./min., C_{H_2O} + 0.16 ml./min., U_{osm} 265 mOsm./L., and osmolar U/P ratio 0.91. Mean values on cortisone therapy were 1.47 ml./min., + 0.82 ml./min., 189 mOsm./L., and 0.65, respectively.

not excessively prolonged. A moderate rise in urinary osmolarity followed hypertonic saline infusion and a normal antidiuresis to Pitressin was evident despite increased rates of solute excretion during this period. One hundred milligrams of hydrocortisone hemisuccinate* given intravenously over a 90-minute period elevated C_{H_2O} to higher levels than occurred initially with hydration, but this increase was related to augmented excretion of solute rather

*Supplied through the courtesy of Dr. C. J. O'Donovan, The Upjohn Company, Kalamazoo, Mich.

than to greater diuresis of solute-free water since urinary osmolarity was not further depressed. The antidiuretic effect of 1.0 mg. nicotine was less pronounced following administration of hydrocortisone.

Measurement of the volume and osmolarity of urine specimens obtained periodically throughout this study on ad libitum dietary and fluid intakes (Table I) demonstrated slight decline in urine flow with the development of myxedema, but the lowest volumes and highest urinary osmolarities of the entire study were obtained during thyroid administration. The addition of cortisone to the therapeutic program resulted in increased urine flow and decreased osmolarity. The effect of cortisone therapy on excretion of osmolar water (C_{osm}) and solute-free water is shown in Fig. 1. Although the number of observations were too few for definite conclusions, increased excretion of solute-free water was observed during cortisone therapy which exceeded that determined by the rates of solute excretion.[17] These findings could be attributed either to increased water intake or decreased solute-free water reabsorption by the distal tubules.

Concentration tests performed periodically throughout this study by overnight dehydration for periods of 11 to 16 hours demonstrated maximal urinary osmolarities of 457 and 419 mOsm./L. when the patient was untreated, and 447 mOsm./L. during stilbestrol and thyroid therapy. Two specimens collected during cortisone therapy 12 to 18 hours after the last dose of cortisone taken the preceding afternoon were recorded at 402 and 448 mOsm./L., respectively. However, 50 mg. cortisone administered in the morning following overnight dehydration decreased urinary osmolarity despite continued water deprivation (Table II, *D*). On one occasion, concentrating capacity was impaired after prolonged cortisone therapy even though cortisone was withdrawn for a 3-day period (Table II, *E*).

CASE 2.—M. V. (NEDH J66423),* a 31-year-old housewife with diabetes mellitus of 16 years' duration, went into labor during the thirtieth week of pregnancy and, on Oct. 3, 1955, delivered a viable infant which died shortly after birth. She subsequently developed persistent hypotension and severe hypoglycemia and failed to lactate post partum. Laboratory studies revealed serum Na of 132 and serum K of 4.0 mEq./L., 24 hour I^{131} uptake 26 per cent, B.M.R. −19 per cent, and urinary 17-OHCS and 17-KS of 2.2 mg. and 6.1 mg. per 24 hours, respectively.

The patient was treated intermittently over a 2-week period with adrenal steroids and ACTH gel. Polydipsia and polyuria became apparent with steroid therapy as well as during ACTH stimulation of the adrenal cortex and declined when steroids were withdrawn (Table III). Urine flow exceeded fluid intake on the first day of steroid administration. Diabetes mellitus recurred but was controlled with insulin and glycosuria did not contribute to polyuria during steroid therapy. A test of neurohypophyseal function performed 54 hours after the last dose of ACTH (Table IV) demonstrated a moderate antidiuresis with administration of nicotine and hypertonic saline, and a slightly greater response to 25 mu. Pitressin, indicative of some neurohypophyseal activity.

On Oct. 26, 1955, when the patient received 25 mg. cortisone by mouth twice daily, urinary osmolarity was 135 mOsm./L. in a specimen collected at 4 P.M. and, despite restriction of fluids after 10 P.M., urine voided at 5 A.M. equaled only 89 mOsm./L. These low-urinary

*We wish to express our appreciation to Dr. Robert Bradley, Joslin Clinic, Boston, Mass., for permission to study this patient and to include in this report the laboratory studies performed under his direction.

osmolarities indicated that a significant portion of the urinary volume was composed of solute-free water and that a marked solute diuresis was not responsible for the urine flow of 4.9 L. observed that day. These urinary osmolarities were much less than the value obtained prior to water administration two days earlier, when the patient was not receiving steroid therapy (see Table IV).

TABLE III. EFFECT OF ADRENAL STEROIDS ON URINE VOLUME IN PATIENT M. V.

OCT. 1955	FLUID INTAKE (L.)	URINE VOL. (L.)	CORTISONE (P.O. MG.)	HYDROCORTISONE (I.V. MG.)	ACTH GEL (I.M. MG.)	17-OHCS (MG.)	17-KS (MG.)
11	1.2*	1.4				2.2	6.1
13	1.9	2.4	50	100			
14	3.8	3.1	25				
15	5.2	3.2	25				
16	3.4	3.6	25				
17	4.1	4.5					
18	2.8	3.0†				0.0	
19	3.9	1.9			80	0.0	6.9
20	4.5	2.8			80	13.8	13.8
21	2.8	3.2			80	27.6	21.5
22	4.2	6.6			40	11.0	14.3
23	2.8	3.1					
24	4.5	3.6‡		100			
25	1.8	1.5	25				
26	2.7	4.9	50				

*Fluids administered I.V. on this day. Remaining values represent oral intakes.
†Four units aqueous Pitressin s.c.
‡Two hundred and thirty milliequivalents NaCl I.V., 60 mu. Pitressin IV.

TABLE IV. STUDIES OF NEUROHYPOPHYSEAL FUNCTION IN PATIENT M. V.

TIME (MIN.)	TREATMENT	V (ML./MIN.)	U_{OSM} (mOsm./L.)	P_{OSM} (mOsm./L.)	C_{OSM} (ML./MIN.)	C_{H_2O} (ML./MIN.)	C_{CR} (ML./MIN.)
0- 75	20 ml. water/Kg. p.o.*						
75- 90		2.3	165	271	1.4	+0.9	26
105-120		4.3	167		2.6	+1.7	51
120	Nic. 0.5 mg. I.V.						
150-165		2.3	221		1.9	+0.4	46
190-205		1.6	182		1.1	+0.5	32
205-235	3% saline 450 ml. I.V.	2.0†	256	284	1.8	+0.2	33
300	Water 700 ml. p.o.						
330-345		2.3	207	283	1.7	+0.6	31
360	Pit. 25 mu. I.V. 300 ml. water p.o.						
390-405		2.3	271	275	2.2	+0.1	30

The patient was allowed fluids ad libitum prior to this study.
*Hydration maintained by administration of water orally or as 5 per cent dextrose in water intravenously at a rate equal to urine flow. Water administration was discontinued prior to saline infusion to decrease the fluid balance to approximately 10 ml./Kg. in order to minimize dilution of the hypertonic saline in the extracellular fluid compartment.
†Minimum urine flow during or after 3 per cent saline infusion.

DISCUSSION

Post-partum pituitary necrosis is a common cause of anterior pituitary insufficiency in the female,[18] but associated posterior lobe destruction and clinical diabetes insipidus are extremely rare. Leaf and associates[5] suggested that the lack of polyuria in combined anterior and posterior pituitary insufficiency may be due to a spontaneous reduction in solute intake and excretion

rather than to loss of hormones specifically influencing water excretion. However, these authors did not fully evaluate certain basic considerations which may have influenced proper interpretation of their data. Since diabetes insipidus was caused by eosinophilic granuloma which frequently invades the infundibular stalk and hypothalamus,[19] complete destruction of hypothalamic centers for ADH production may have been anticipated, and the low urinary osmolarities observed in their patient suggested that such degeneration had occurred. In the absence of ADH, urinary total solute concentration should assume a minimal value determined principally by the rate of distal tubular reabsorption of solute at normal rates of urine flow, and diuretic properties of various hormones may not be demonstrable since the ability to increase water excretion independently of solute excretion would require some capacity to further dilute the urine by decreasing the reabsorption of solute-free water. In addition, the patient received a relatively low salt intake throughout the study, so that increased aldosterone secretion may also have contributed to the low urinary osmolarity by increasing tubular reabsorption of sodium.

The patients in this report excreted moderately concentrated urines in the absence of cortisone therapy suggesting some neurohypophyseal function sufficient to prevent marked polyuria; antidiuretic responses to relatively small doses of nicotine were demonstrable in both subjects. Adrenal steroids increased water excretion in both patients, and in one subject, solute-free water diuresis with hydration was improved and the profound antidiuretic response to nicotine was suppressed. These findings implied a possible relationship between inhibitory effects of cortisone on ADH secretion and the improvements in water excretion observed. The fact that dilution of the urine and diuresis of solute-free water were accentuated, despite standard hydration and comparable depression of serum osmolarity in a patient in whom osmoreceptor function was not demonstrable, suggested that cortisone had inhibited an antidiuretic mechanism in the hypothalamus not regulated by extracellular tonicity[20] which may have been responsible for the delayed diuresis. This hypothesis is corroborated by the demonstration that adrenal steroids inhibit the antidiuretic response to nicotine but not to hypertonic saline in normal subjects,[21] as well as by the observation that cortisone decreased urinary concentrating ability in this patient during fluid restriction when osmoreceptor activity is presumably accentuated.

The depressed water diuresis and extreme sensitivity to nicotine which were evident when the patient developed myxedema were probably related to a more advanced state of adrenocortical insufficiency rather than to the level of thyroid activity, since therapy with desiccated thyroid did not reverse these abnormalities and may have actually accentuated them.

The failure of urinary osmolarity to increase upon withdrawal of cortisone in May, 1955, and the fairly normal water diuresis apparent despite withdrawal of cortisone therapy, were difficult to interpret, but may have been due to alterations in hypothalamic mechanisms for ADH production, or spontaneous or induced changes in renal function. The decrease in solute excretion noted during this period and the greater tolerance to nicotine suggested

that increased secretion of aldosterone may have been responsible for these findings, and there is some evidence that aldosterone may have hydrocortisone-like effects on water metabolism.[22] Since water administration resulted in maximal dilution of the urine in this study, a water-diuretic effect of hydrocortisone may not have been anticipated, but an inhibitory effect of this hormone on nicotine-induced ADH release was clearly demonstrable.

These studies suggest that the oliguria and delayed water diuresis of anterior pituitary insufficiency may be determined, at least in part, by persistent secretion of ADH from the neurohypophysis. Damage to or surgical removal[23] of both anterior and posterior lobes of the pituitary gland results in a variable degree of polyuria probably determined in part by the adequacy of secretion of ADH from hypothalamic portions of the neurohypophysis on the one hand, and the level of adrenal cortical secretion on the other. One mechanism by which adrenal steroids increase water excretion in combined pituitary insufficiency may be through inhibitory effects of these hormones on secretion of ADH from the hypothalamus.

Although this study had dealt primarily with the endocrine control of water excretion in man, consideration must also be given to the dominant role of the kidney in determining the ultimate composition of the urine. Some studies have shown that slightly hypertonic urines may be formed in the absence of antidiuretic hormone when glomerular filtration rate is profoundly depressed[24, 25]; however, others[26] have been unable to demonstrate this phenomenon.

Although creatinine clearances were moderately depressed in both patients in this report, it is unlikely that glomerular filtration rate was sufficiently lowered to account for the decrease in polyuria and moderate concentration of the urine on this basis. The normal water diuresis noted with cortisone therapy (Table II, *D*) was probably not due to alterations in glomerular function since thyroid therapy alone (Table II, *C*) did not improve water diuresis even though creatinine clearance was elevated to comparable levels, and there is a large body of evidence indicating that the diuretic effect of cortical hormones is predominantly at the distal tubular site for water reabsorption.[27]

Moderate hypotonicity of the urine during mannitol diuresis has been demonstrated in patients with diabetes insipidus simultaneously receiving small doses of Pitressin.[16] Although this study implies that absence of antidiuresis to hypertonic saline in one patient in this report, as well as in two other patients described elsewhere,[7] may have been due to increased excretion of solute masking the effect of released ADH, the fact that antidiuresis also was not apparent in one study in which solute diuresis did not occur (Table II, *D*), and that the effects of small doses of Pitressin were not altered despite increased excretion of solute in two other instances, suggests that factors other than those inherent in the kidney may have influenced these results.

Although these findings make tenable the hypothesis that adrenal steroids influence water excretion by altering neurohypophyseal secretory activity,

proof of this theory must await more detailed investigation of the influence of the endocrine system on renal function and water excretion in man.

SUMMARY AND CONCLUSIONS

The relationship of anterior pituitary function to polyuria was evaluated in two patients with post-partum pituitary necrosis (Sheehan's syndrome) and combined anterior and posterior pituitary insufficiency who provided evidence for residual secretion of ADH with appropriate neurohypophyseal stimulation. These studies permit the tentative conclusion that amelioration of polyuria in combined pituitary insufficiency may be due, in some instances, to persistent secretion of ADH from viable hypothalamic neurosecretory tissue. At least one anterior pituitary factor responsible for the disturbed water metabolism of posterior pituitary insufficiency appears to be an intact pituitary-adrenocortical axis. The hypothesis is made that hydrocortisone-like adrenal steroids may produce polyuria and promote solute-free water diuresis by inhibiting an antidiuretic mechanism for ADH release not regulated by extracellular tonicity located in the hypothalamic portion of the neurohypophysis.

REFERENCES

1. Richter, C. P.: Experimental Diabetes Insipidus: Its Relation to the Anterior and Posterior Lobes of the Hypophysis, Am. J. Physiol. **110**: 439, 1934-35.
2. Mahoney, W., and Sheehan, D.: The Effect of Total Thyroidectomy Upon Experimental Diabetes Insipidus in Dogs, Am. J. Physiol. **112**: 250, 1935.
3. Schweizer, M., Gaunt, R., Zinken, N., and Nelson, W. O.: The Role of the Adrenal Cortex and the Anterior Pituitary in Diabetes Insipidus, Am. J. Physiol. **132**: 141, 1941.
4. de Bodo, R. C., Schwartz, I. L., Greenberg, J., Kurtz, M., Earle, D. P., Jr., and Farber. S. J.: Effect of Growth Hormone on Water Metabolism in Hypophysectomized Dogs, Proc. Soc. Exper. Biol. & Med. **76**: 612, 1951.
5. Leaf, A., Mamby, A. R., Rasmussen, H., and Marasco, J. P.: Some Hormonal Aspects of Water Excretion in Man, J. Clin. Invest. **31**: 914, 1952.
6. Dingman, J. F., and Thorn, G. W.: Cortisone Inhibition of ADH Secretion From the Neurohypophysis, J. Clin. Endocrinol. **15**: 871, 1955.
7. Dingman, J. F., Benirschke, K., and Thorn, G. W.: Studies of Neurohypophyseal Function in Man: Diabetes Insipidus and Psychogenic Polydipsia, Am. J. Med. **23**: 226, 1957.
8. Wesson, L. G., Jr., and Anslow, W. P., Jr.: Effect of Osmotic and Mercurial Diuresis on Simultaneous Water Diuresis, Am. J. Physiol. **170**: 255, 1952.
9. Reddy, W. J., Jenkins, D., and Thorn, G. W.: Estimation of 17-hydroxycorticoids in Urine, Metabolism **1**: 511, 1952.
10. Drekter, I. J., Heisler, A., Scism, G. R., Stern, S., Pearson, S., and McGavick, T. H.: The Determination of Urinary Steroids. I. The Preparation of Pigment Free Extracts and a Simplified Procedure for the Estimation of Total 17-ketosteroids, J. Clin. Endocrinol. **12**: 55, 1952.
11. Klinefelter, H. F., Jr., Albright, F., and Griswold, G. C.: Experience With a Quantitative Test for Normal or Decreased Amounts of Follicle Stimulating Hormone in Urine, J. Clin. Endocrinol. **3**: 529, 1943.
12. Barker, S. B., Humphrey, M. J., and Soley, M. H.: The Clinical Determination of Protein Bound Iodine, J. Clin. Invest. **30**: 55, 1951.
13. Bosnes, R. W., and Taussky, H. H.: The Colorimetric Determination of Creatinine by the Jaffe Reaction, J. Biol. Chem. **158**: 581, 1945.
14. Schales, O., and Schales, S. S.: Determination of Chloride in Biological Fluids, J. Biol. Chem. **140**: 879, 1941.
15. Rosenbaum, J. D., Nelson, W. P., III, Strauss, M. B., Davis, R. K., and Rossmeisl, E. C.: Variation in the Diuretic Response to Ingested Water Related to the Renal Excretion of Solutes, J. Clin. Invest. **32**: 394, 1953.
16. de Wardener, H. E., and del Greco, F.: The Influence of Solute Excretion Rate on the Production of a Hypotonic Urine in Man, Clin. Sc. **14**: 715, 1955.

17. Zak, G. A., Brun, C., and Smith, H. W.: Mechanism of Formation of Osmotically Concentrated Urine During the Antidiuretic State, J. Clin. Invest. **33**: 1064, 1954.
18. Sheehan, H. L.: The Incidence of Postpartum Hypopituitarism, Am. J. Obst. & Gynec. **68**: 202, 1954.
19. Thannhauser, S. J.: Lipoidoses: Diseases of the Cellular Lipid Metabolism, New York, 1940, Oxford University Press.
20. Leaf, A., and Mamby, A. R.: An Antidiuretic Mechanism not Regulated by Extracellular Tonicity, J. Clin. Invest. **31**: 60, 1952.
21. Dingman, J. F., and Despointes, R. H.: Adrenal Steroids and Water Metabolism, J. Clin. Invest. **36**: 882, 1957.
22. Dingman, J. F., Finkenstaedt, J. T., Laidlaw, J. C., Renold, A. E., Jenkins, D., Merrill, J. P., and Thorn, G. W.: Influence of Intravenously Administered Adrenal Steroids on Sodium and Water Excretion in Normal and Addisonian Subjects, Metabolism. (In press.)
23. Dingman, J. F., Jessiman, A. G., Despointes, R. H., Hammond, W. G., Matson, D. D., Emerson, K., Jr., and Moore, F. D.: Mechanisms of Polyuria in Hypophysectomized Man, Proceedings Endocrine Society, New York, May, 1957.
24. Berliner, R. W., and Davidson, D. G.: Production of Hypertonic Urine in the Absence of Pituitary Antidiuretic Hormone, J. Clin. Invest. **36**: 1416, 1957.
25. del Greco, F., and de Wardener, H. C.: The Effect on Urine Osmolarity of a Transient Reduction in Glomerular Filtration Rate and Solute Output During a "Water" Diuresis, J. Physiol. **131**: 307, 1956.
26. Leaf, A., Kerr, W. S., Jr., Wrong, O., and Chatillon, J. Y.: Effect of Gradual Compression of the Renal Artery on Water and Solute Excretion, Am. J. Physiol. **179**: 191, 1954.
27. Gaunt, R., Birnie, J. H., and Eversole, W. J.: Adrenal Cortex and Water Metabolism, Physiol. Rev. **29**: 281, 1949.

THE EFFECT OF CORTISONE AND HYDROCORTISONE ON HEPATIC EXCRETORY FUNCTION

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INTEREST in a possible choleretic action of adrenal glucocorticoids has been stimulated by clinical observations that the jaundice of hepatitis often decreases rapidly following the administration of adrenocorticotrophic hormone or cortisone.[1-3] Patterson, Dingman, Shwachman, and Thorn[4] studied this problem directly and concluded that cortisone possesses cholerectic and hydrocholeretic properties. Since the evidence presented in these studies did not appear to be entirely conclusive, we have investigated the effect of cortisone and hydrocortisone on several different aspects of hepatobiliary function.

Effect of Cortisone on Bromsulphalein Clearance.—

Method: In 10 healthy volunteers plasma clearance of Bromsulphalein (C_{BSP}) was measured before and after the oral administration of 50 mg. of cortisone 4 times a day for 3 days. The last dose of cortisone was taken 2 hours before the test; otherwise, the subject was fasting for 6 hours. C_{BSP} was determined by methods described previously[5] except a calibrated pump was used to infuse the dye. Plasma BSP determinations were done in duplicate using the Coleman Junior spectrophotometer.[6]

Results: In 8 subjects, C_{BSP} decreased following cortisone, in one it remained unchanged, and in one clearance increased 96 ml./minute (Table I). The group mean control C_{BSP} was 535 ml./min./1.73 M.^{2} and that after cortisone was 450 ml./min./1.73 M.^{2}, a decrease of 15.9 per cent. Statistical evaluation of these paired data revealed that the change is significant ($t = 2.82$; $p = 0.02$). In the test performed immediately after the period of cortisone administration, rising plasma levels of BSP occurred in 3 subjects. No such progressive increase in plasma dye level was encountered in any control observation. In subject J. A., a second control study 2 weeks after cortisone had been discontinued also showed constant plasma levels of BSP (Fig. 1).

Effect of Cortisone on Hepatic Uptake of I^{131}-Labeled Rose Bengal.—

Methods: In 8 healthy volunteers, hepatic uptake of rose bengal labeled with I^{131}† was determined by the method of Taplin and associates.[7] A collimated sodium iodide crystal

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TABLE I. EFFECT OF ORAL CORTISONE ON PLASMA CLEARANCE OF BSP

SUBJECT	CONTROL			POSTCORTISONE*		
	INFUSION RATE BSP (MG./MIN./M.²)	MEAN PLASMA LEVEL BSP (MG. PER CENT)	MEAN C_{BSP} (ML./MIN./1.73 M.²)	INFUSION RATE BSP (MG./MIN./M.²)	MEAN PLASMA LEVEL BSP (MG. PER CENT)	MEAN C_{BSP}† (ML./MIN./1.73 M.²)
P. K.	2.73	1.05	448	2.71	1.20	389
K. J.	2.80	1.00	485	2.67	0.95	488
S. S.	2.93	1.00	507	2.85	1.13	433
T. D.	2.70	0.93	503	2.63	0.78	599
A. H.	2.82	0.62	594	2.76	0.94	503
J. G.	2.73	0.63	754	2.65	0.73	627
A. R.	2.79	0.89	542	2.74	1.03	463
T. H.	2.74	0.81	587	2.69	1.36	339
M. A.	2.66	0.84	546	2.84	1.41	340
J. A.	2.73	1.24	385	2.80	1.58	314
Mean			535 ±99.2			450 ±108

*Two hundred milligrams daily for 3 days.
†$t = 2.82$; $p = 0.02$.

scintillation counter was placed over the upper edge of the liver in order to minimize the effect of radiation from dye collecting in the gall bladder. Indelible ink skin marks were used to place the counter in the same position on successive determinations. The counting head was connected through a ratemeter to an Esterline-Angus graphic recorder. Tracer doses of I^{131}-labeled rose bengal (5 μc) containing 1 mg. or less of rose bengal was given intravenously and continuous recording was done for 1½ to 2 hours thereafter. Two control studies were done at 3- to 5-day intervals after which 50 mg. of cortisone was given 4 times a day for 3 days, and hepatic uptake of labeled rose bengal was measured again approximately 2 hours after the last dose.

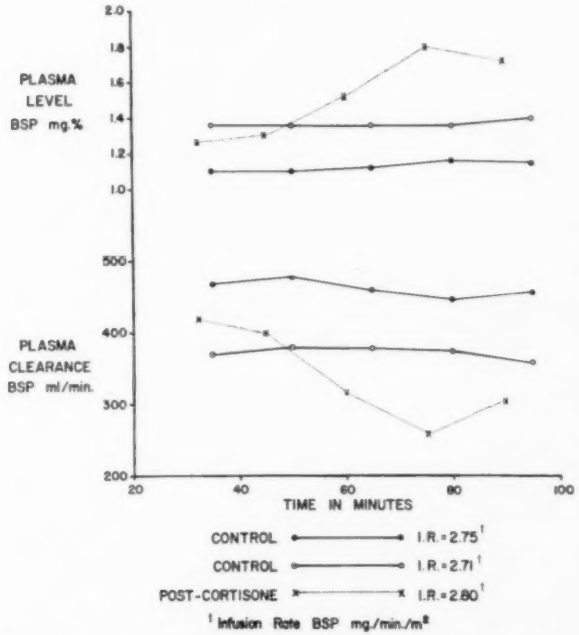

Fig. 1.—Plasma clearance BSP following cortisone. Patient J. A. Rising plasma levels of BSP and decreasing C_{BSP} (x···x) following 3 days of oral cortisone. Control studies before (•——•) and 2 weeks after cortisone (○——○) reveal constant plasma levels.

Results: In 3 subjects the tests were technically unsatisfactory. In 4 subjects the maximum values of counts per minute after cortisone were less than the means of the maximum values before cortisone. In one the maxima were similar. In Fig. 2, the group means of the control and the postcortisone observations are plotted against time.

In 7 of the subjects, plasma disappearance rates were calculated according to the method of Ingelfinger and associates.[8] After injection of the dye, blood samples were drawn at 4, 6, 8, 10, and 12 minutes from the opposite arm and placed in oxalate bottles. Samples obtained after 12 minutes did not have enough radiation to insure accurate counting. Following centrifugation, 2 ml. of plasma was pipetted into small test tubes and placed in a scintillation

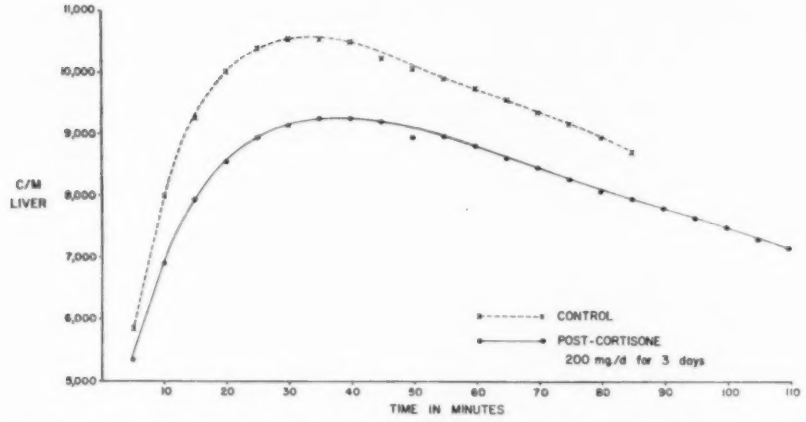

Fig. 2.—Counts over the liver following the injection of I^{131}-labeled rose bengal. Hepatic uptake-excretion curves of I^{131}-labeled rose bengal before (x - - - x) and after (●——●) oral cortisone. Each point on the line represents the mean counts per minute of the 5 subjects.

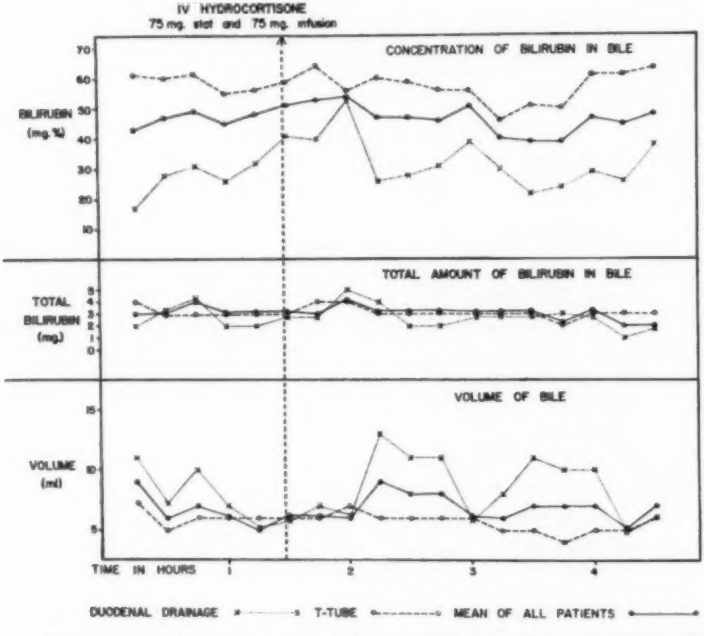

Fig. 3.—The mean values before and after IV hydrocortisone of volume output, bilirubin concentration, and bilirubin output in bile obtained from 6 patients with T tubes in the common ducts and from 4 cholecystectomized patients by duodenal drainage. The heavy black line represents the mean for all 10 patients.

well counter. The percentage disappearance rates calculated for each patient before and after cortisone were almost identical (Table II). The mean of the control observations for the group was 8.2 per cent and that after cortisone was 8.1 per cent.

TABLE II. PLASMA DISAPPEARANCE RATE OF I^{131}-LABELED ROSE BENGAL

SUBJECT	CONTROL (PER CENT/MIN.)	CONTROL (PER CENT/MIN.)	MEAN CONTROL (PER CENT/MIN.)	POSTCORTISONE* (PER CENT/MIN.)
P. H.	7.2	7.1	7.2	7.2
D. R.	7.3	7.6	7.5	6.9
D. B.	7.1	7.4	7.3	7.5
L. R.	10.3	10.7	10.5	9.9
N. A.	--	8.7	8.7	8.8
A. H.	8.5	9.0	8.8	9.9
C. M.	7.2	7.4	7.3	6.2
Mean			8.2	8.1

*Two hundred milligrams daily for 3 days.

Effect of Intravenous Hydrocortisone on Excretion of Bile and Bilirubin.—

Methods: Intravenous hydrocortisone was given to 6 patients who had T tubes in the common bile ducts and were recovering from uncomplicated cholecystectomy for cholelithiasis. They were not tested for at least 7 days after cholecystectomy since maximal T tube flow is not established until then.[9] All were afebrile and had fasted for 8 hours. With the patients supine, bile was collected from the T tubes in consecutive 15-minute periods by gravity drainage. After a control period of 1½ hours, 75 mg. of hydrocortisone hemisuccinate sodium* was given in one injection intravenously, and 75 mg. dissolved in 300 ml. normal saline was infused at a constant rate over a 3-hour period. Volume output, bilirubin concentration, and total bilirubin output were measured.

Bilirubin determinations were done according to the oxidation method of Malloy and Evelyn,[10] using hydrogen peroxide as the oxidant. In order to obtain reproducible results we found it necessary to control temperature as well as time; therefore, the reaction was allowed to proceed for one hour in a water bath at 30° C. Samples were read in duplicate at 660 mμ in a Coleman Junior spectrophotometer.

In 4 patients who had had cholecystectomy several months previously and were without evidence of hepatobiliary disease bile was collected by duodenal drainage. The tip of a double lumen tube was placed in the distal duodenum under fluoroscopic control so that gastric and duodenal contents could be collected separately. Continuous aspiration was carried out with a suction pump at minus 90 mm. Hg. Patency of both lumens was checked every 15 minutes, and the position of the tube was verified periodically with the fluoroscope. Except for the method of bile collection, the experimental procedures were identical to those used in measuring T tube output.

Results: The average values for volume output, bilirubin concentration, and bilirubin output did not change appreciably after hydrocortisone (Fig. 3). The mean volume output of bile for the entire group before hydrocortisone was 6.8 ml./15 minutes and after hydrocortisone was 7.1 ml./15 minutes. The respective means for bilirubin concentration were 43.8 and 44.2 mg./100 ml.; and 3.0 mg. and 3.1 mg./15 minutes for total bilirubin output. Four patients had gradually increasing concentrations of bilirubin after hydrocortisone, 2 had gradually decreasing levels, and 4 evidenced no change. In a like manner, 2 patients had increased volume of bile after hydrocortisone, 4 a decreasing

*Solu-Cortef; The Upjohn Company, Kalamazoo, Mich.

volume, and in 4 the output was unchanged. In only 2 patients did the factors of volume and concentration vary in the same direction, and in these both decreased.

DISCUSSION

Hepatic excretory activity is considered to have 3 major components: the removal of substances from blood, storage in the liver for a variable period, and excretion in bile. These same components presumably participate in the excretion of exogenous substances, e.g., certain dyes, as well as endogenous material, e.g., bilirubin and water. Our results do not suggest that these components of hepatic excretory activity are accelerated by the oral administration of cortisone or the intravenous administration of hydrocortisone in the doses employed.

The clearance of Bromsulphalein and the percentage disappearance rate of I^{131}-labeled rose bengal from the plasma were not increased following cortisone. Indeed, there was some evidence that C_{BSP} is diminished after the administration of this steroid. The finding of Meyers and Taylor[11] that C_{BSP} is unaffected by the prior administration of a single oral dose of 200 mg. of cortisone also indicates that cortisone does not affect this component of hepatic excretory activity.

The counts obtained over the liver during the uptake, storage, and excretion of rose bengal I^{131} represent the algebraic sum of these processes. Repeat determinations on the same patient are valid only if the counting technique is absolutely unchanged. Since this is difficult to accomplish a precise physiologic interpretation of the uptake-excretion curves must be done with caution. Gross changes in the uptake-excretion curves did not occur following cortisone administration. The lower mean maximum value of counts found after cortisone is subject to several possible explanations, one being that a more rapid excretion of rose bengal prevented accumulation of the dye in the liver. This possibility, however, is not supported by the slopes of the final portion of the curves. The trends of these slopes, when inspected with due regard to the maximum value achieved, are grossly similar. To the extent therefore that the inclination of these slopes is determined by the hepatic excretion of the dye, no evidence of increased excretory activity after cortisone is adduced.

Total bilirubin output, bilirubin concentration, and volume output of bile were unaffected by intravenous hydrocortisone in patients with T tubes in the common ducts as well as in other cholecystectomized patients from whom samples were collected by duodenal drainage. Collection by these means is subject to error since a variable amount of the bile or duodenal content may escape aspiration. In general, however, losses of this type have been shown to be very small.[9]

The findings we have reported are at variance with those of Patterson and associates[4] but several factors in their experimental design may account for the difference. (1) The gall bladder was present in 6 of 7 patients studied by these authors; in the presence of this viscus with its independent capacity to store, concentrate, and evacuate bile, accurate assessment of hepatic bile output

would appear impossible. (2) A single lumen tube was used to collect duodenal specimens. Volumetric measurement of pure duodenal content is itself of limited value for the purpose of measuring bile output because of the presence of pancreatic and intestinal juices, but the use of a single lumen tube permits additional contamination by exceedingly variable quantities of gastric juice. (3) Bilirubin determinations were done by the diazo method of Malloy and Evelyn.[12] This method is not well suited for the estimation of bilirubin in bile because the diazo reagent does not couple with biliverdin.[13] Upon exposure to light and air, bilirubin in bile is rapidly oxidized to biliverdin and falsely low values for bilirubin excretion may be obtained with the diazo method.

Methods for measuring hepatic secretory activity in man are not ideal, and our techniques are subject also to uncontrollable influences, i.e., the role of extrahepatic sites in determining C_{BSP}, or incomplete collection of biliary output by T tube drainage. The uniformly negative results obtained by the several different approaches to hepatic excretory function would, however, tend to support their validity and justify the suggestion that cortisone or hydrocortisone does not induce choleresis or hydrocholeresis under normal conditions.

SUMMARY AND CONCLUSIONS

1. Oral cortisone did not accelerate the plasma clearance of Bromosulphalein or the percentage disappearance rate of I^{131}-labeled rose bengal from the plasma.

2. The hepatic uptake-excretion curves of rose bengal I^{131} did not change to suggest increased hepatic excretion after oral cortisone.

3. Intravenous hydrocortisone did not alter the volume, the bilirubin concentration, or total bilirubin output within a period of 3 hours.

4. No evidence for a choleretic or hydrocholeretic action of cortisone or hydrocortisone was found.

ADDENDUM

In a recent publication (New England J. Med. **257**: 62, 1957) Shay and Sun also report that they are unable to demonstrate any increased output of bilirubin following the intravenous administration of 100 mg. of hydrocortisone. Ten patients with T tubes in the common bile ducts were studied.

REFERENCES

1. Colbert, J. W., Jr., Holland, J. F., Heissler, I., and Knowlton, M.: Use of ACTH in Acute Viral Hepatitis, New England J. Med. **245**: 172-177, 1951.
2. Evans, A. S., Sprinz, H., and Nelson, R. S.: Adrenal Hormone Therapy in Viral Hepatitis. I. The Effect of ACTH in the Acute Disease, Ann. Int. Med. **38**: 1115-1133, 1953. Adrenal Hormone Therapy in Viral Hepatitis. II. The Effect of Cortisone in the Acute Disease, Ibid. **38**: 1134-1147, 1953. Adrenal Hormone Therapy in Viral Hepatitis. III. The Effect of ACTH and Cortisone in Severe and Fulminant Cases, Ibid. **38**: 1148-1159, 1953.
3. Sborov, V. M., Giges, B., Plough, I. C., and Mandel, W.: ACTH Therapy in Acute Viral Hepatitis, J. Lab. & Clin. Med. **43**: 48-57, 1954.
4. Patterson, P. R., Dingman, J. F., Shwachman, H., and Thorn, G. W.: Choleretic Action of Cortisone, New England J. Med. **251**: 502-508, 1954.
5. Meyers, J. D., and Hickam, J. B.: An Estimation of the Hepatic Blood Flow and Splanchnic Oxygen Consumption in Heart Failure, J. Clin. Invest. **27**: 620-627, 1948.

6. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J.: The Estimation of Hepatic Blood Flow in Man, J. Clin. Invest. **24**: 890-897, 1945.
7. Taplin, G. V., Meredith, O. M., Jr., and Kade, H.: The Radioactive (I^{131}-tagged) Rose Bengal Uptake-Excretion Test for Liver Function Using External Gamma-ray Scintillation Counting Techniques, J. LAB. & CLIN. MED. **45**: 665-678, 1955.
8. Ingelfinger, F. J., Bradley, S. E., Mendeloff, A. I., and Kramer, P.: Studies With Bromsulphalein. I. Its Disappearance from the Blood After a Single Intravenous Injection, Gastroenterology **11**: 646-657, 1948.
9. Rundle, F. F., Cass, M. H., Robson, B., and Middleton, M.: Bile Drainage After Choledochostomy in Man, With Some Observations on Biliary Fistula, Surgery **37**: 903-910, 1955.
10. Malloy, H. T., and Evelyn, K. A.: Oxidation Method for Bilirubin Determinations in Bile and Meconium With the Photoelectric Colorimeter, J. Biol. Chem. **122**: 597-603, 1938.
11. Meyers, J. D., and Taylor, W. J.: The Effects of Cortisone and ACTH on Splanchnic Circulation and Metabolism, J. Clin. Invest. **31**: 651, 1952.
12. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119**: 481-490, 1937.
13. Lemberg, R., and Legge, J. W.: Hematin Compounds and Bile Pigments, New York, 1949, Interscience Publishers, Inc., p. 155.

ANTI-INFLAMMATORY ACTIVITY OF COMPOUNDS OBTAINED FROM EGG YOLK, PEANUT OIL, AND SOYBEAN LECITHIN

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WITH THE TECHNICAL ASSISTANCE OF JOHN R. RYAN

THE presence of an antiallergic factor in peanut oil, soybean lecithin, and egg yolk has been reported by Long[1, 4] and by Coburn and associates.[2, 3] Long and Martin[1] found that peanut oil significantly depressed the sensitivity of BCG (baeille Calmette Guérin) infected guinea pigs to tuberculin. Fractionation of peanut oil produced material which in doses of 6×10^{-8} Gm./Kg. of body weight reduced sensitivity to tuberculin, while 5 mg./Kg. of body weight of cortisone was required to effect a similar reduction.[1]

Coburn, believing that the conditioning factors of rheumatic fever are a combination of nutrition and heredity, studied the nutritional aspects of the disease.[2, 5] In these studies an inverse relation between intake of eggs and rheumatic fever was found.[2] This finding prompted him and his associates to investigate the role of eggs and extracts of egg in rheumatic fever both clinically and in the laboratory. They devised an anaphylaxis assay performed in the joints of guinea pigs and reported a decrease in swelling after feeding of an alcohol soluble extract of egg yolk. Clinical studies, using the same extract, also showed some reduction of rheumatic recrudescences in children.[2]

The above findings, particularly the very low concentrations of peanut oil extract needed to reduce tuberculin sensitivity, prompted us to investigate this problem further. The anti-inflammatory activity of various fractions of peanut oil, soybean lecithin, and egg yolk was studied using a variety of anti-inflammatory assays.

MATERIALS AND METHODS

Crude samples of egg yolk, soybean lecithin, and peanut oil as well as a number of partially purified and pure crystalline compounds derived therefrom were supplied by Drs. Kuehl and Jacob.[6*]

Guinea Pig Joint Anaphylaxis Assay.—Albino guinea pigs of both sexes weighing between 250 to 350 grams were used. Approximately 4,000 guinea pigs were used in this study. In individual experiments the weight of the animals did not vary more than 50 grams. The animals were kept in an air-conditioned room, 8 to 10 per cage, and were allowed free access to water, Purina Rabbit Chow, and fresh cabbage.

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*We are indebted to Dr. F. A. Kuehl, Jr., and Dr. T. Jacob, Merck Sharp & Dohme Research Laboratories (Division of Merck & Company, Inc.), Rahway, N. J., for supplying us with all the compounds used in these studies.

Swelling of joints in guinea pigs was produced by a local joint anaphylaxis reaction. Both hind knee joints were injected with 0.05 ml. of anti-egg albumin serum. About 15 minutes later the right joint was injected with 0.05 ml. of 0.05 per cent egg albumin and the left joint with 0.05 ml. of normal saline to act as a control. At predetermined time intervals the diameter of each joint was measured with a vernier caliper. The degree of swelling was expressed as the difference in diameter (in millimeters) between right and left leg.

Control experiments were run to determine the difference in diameter of normal right and left hind leg joints of guinea pigs. In normal joints or joints injected with saline or egg albumin without sensitization, no significant difference between right and left leg existed (statistical sign test). The average degree of swelling in sensitized pigs was about 1.3 mm. at 3 to 5 hours and about 0.9 mm. 20 hours after antigen injection. No difference in swelling was seen whether the right or left leg received the specific antigen. Although considerable variation was seen from animal to animal the assay was found satisfactory when 8 to 10 pigs per group were used. Therefore, in our standardized test the minimum number of animals per group was 8. Compounds were considered active if more than 50 per cent of the animals showed a 30 per cent or more reduction in swelling. This simple biologic interpretation was checked several times by statistical tests[7, 8*] and the results coincided at all times.

All drugs were suspended in Merck Aqueous Vehicle No. 1† and administered by intraperitoneal injection except when otherwise noted. A normal control group receiving the "carrier" was run with each experiment. At first animals were pretreated for 2 to 3 days before the assay was carried out; however, it was found that treatment 3 to 6 hours before inflammation was just as efficient and the latter treatment schedule was then adapted for the standard test.

Pellet Assay.—The cotton pellet test employed was a modification of that described by Meier and associates[9] and utilizes cotton pellets impregnated with a nonvirulent culture of *Micrococcus pyogenes* var. *aureus*. The presence of the organism stimulated the inflammatory response and increased the sensitivity of the assay. A minimum of 6 rats was used at each dose level. The test compounds were administered both orally and subcutaneously. Treatment was initiated 1 to 20 days before the cotton pellets were implanted and then continued until the assay was completed.

Evans Blue Assay.—The action of these compounds on early stages of inflammation, as reflected by altered capillary permeability, was measured by the Evans blue technique. In these studies, 2 mm. thick cotton dental pellets (Johnson & Johnson No. 1) were implanted under the skin of rats, and sterile Evans blue (4 mg./Kg.) was injected intravenously immediately thereafter. Animals were sacrificed 3 and 6 hours later and the site surrounding the implanted cotton pellet was examined for the absence or presence of dye. Both the diameter of the stained area and intensity of the dye were taken into account. Here again rats were pretreated with the test compounds from 1 to 28 days.

RESULTS

Guinea Pig Assay.—Using the guinea pig joint assay method, a large number of crude samples of soybean lecithin, egg yolk, and peanut oil were assayed and found to be active in a dose range of 60 mg./Kg. to 0.3 μg/Kg. By fractionation of these crude materials *N*(2-hydroxyethyl)-palmitamide was obtained which was active in minute amounts.[6] Typical examples of the results obtained

*We are indebted to Dr. J. Ciminera, Merck Sharp & Dohme Research Laboratories, West Point, Pa., for performing the statistical tests.

†Composition: sodium chloride (reagent) 0.009 Gm.; benzyl alcohol (reagent) 0.009 Gm.; sodium carboxymethylcellulose 0.005 Gm.; Tween 80 0.004 Gm.; sufficient pyrogen-free water to make 1,000 c.c.

with the active principle are presented in Tables I and II. Similar anti-inflammatory effects were obtained with a purified concentrate from peanut oil reported to be active by Dr. D. A. Long.* It is interesting to note that large doses of these active materials produced no greater anti-inflammatory action than the small doses.

TABLE I. SOME TYPICAL RESULTS WITH CRYSTALLINE FRACTIONS FROM SOYBEAN LECITHIN, PEANUT OIL, AND EGG YOLK IN THE GUINEA PIG JOINT ANAPHYLAXIS ASSAY*

SAMPLE	DOSE (μG/KG.)	NUMBER ANIMALS	MEAN DEGREE SWELLING (MM.)	SIGNIFICANCE P	PER CENT DECREASE
Soybean lecithin	Control	8	0.81	---	---
	0.003	8	0.90	>0.05	---
	0.03	8	0.77	>0.05	5†
	0.3	8	0.47	<0.05	41
	3.0	8	0.49	<0.05	39
	30.0	8	0.37	<0.05	54
Egg yolk	Control	8	0.86	---	---
	0.3	8	0.61	>0.05	29†
	3.0	8	0.29	<0.05	66
	30.0	8	0.15	<0.05	82
Peanut oil	Control	8	0.65	---	---
	0.3	8	0.46	>0.05	29†
	3.0	8	0.29	<0.05	55

*Approximately 1,000 pigs were used in this type of assay.
†Not active.

TABLE II. ANTI-INFLAMMATORY EFFECT OF VARIOUS SYNTHETIC COMPOUNDS IN GUINEA PIG JOINT ANAPHYLAXIS ASSAY (A REPRESENTATIVE STUDY)*

COMPOUND	DOSE QUANTITY IN μG/KG.	NO. ANIMALS	MEAN DEGREE OF SWELLING IN MM. 20 HR. LATER	SIGNIFICANCE P	PER CENT DECREASE AT 20 HR.
Controls	---	8	0.83	---	---
Ethanolamine hydrochloride	0.03	8	0.66	>0.05	20†
	0.3	8	0.71	>0.05	14†
	3.0	8	0.21	<0.05	74
	30	8	0.50	<0.05	39
	3,000	8	0.31	<0.05	62
N(2-hydroxyethyl)-palmitamide	0.03	8	0.60	>0.05	27†
	0.30	8	0.43	<0.05	48
	3.00	8	0.53	<0.05	36
	30	8	0.16	<0.05	80
	300	8	0.50	<0.05	39
	3,000	8	0.40	<0.05	51
Control	---	8	0.98	---	---
Choline chloride	0.30	8	0.73	>0.05	25†
	3.00	8	0.49	<0.05	45
	30	8	0.49	<0.05	45
	300	8	0.37	<0.05	58

*A total of 250 guinea pigs were used in this study.
†Inactive.

N(2-hydroxyethyl)-palmitamide was synthesized[6] and on assay proved to be as active as the crystalline compounds obtained from the above-named crude samples. On splitting the *N*(2-hydroxyethyl)-palmitamide it was found that

*Thanks are due to Dr. D. A. Long, National Institute for Medical Research, London, England, for supplying this sample.

the ethanolamine HCl moiety was active at 3 μg/Kg. concentration, while palmitic acid was inactive (Table II). These experiments were repeated 4 times using a total of 250 guinea pigs. Due to variations inherent in this assay it was not possible to show clearly a difference in activity between the palmitamide and the ethanolamine. On a molecular weight basis, the palmitamide is probably 2.5 times more active than the ethanolamine.

Anti-inflammatory Activity of Related Substances.—After the active fraction from soybean lecithin, peanut oil, and egg yolk was identified, various other related compounds were tested for activity (Table III). As seen in Table III the reduced members of the "choline cycle"[12] were active while the oxidized compounds were inactive. Choline chloride was found to be active at 3 μg/Kg.

TABLE III. COMPARISON OF CHEMICAL STRUCTURE WITH BIOLOGIC ACTIVITY

Ethanolamine	$NH_2CH_2CH_2OH$	Active
Glycine	NH_2CH_2COOH	Inactive
Serine	$CH_2OHCH(NH_2)COOH$	Inactive
Sarcosin	$CH_3NH\ CH_2COOH$	Inactive
Betaine	$(CH_3)_3\overset{+}{N}\ CH_2COOH$	Inactive
Choline	$(CH_3)_3\overset{+}{N}\ CH_2OH$	Active
2-Dimethylaminoethanol	$(CH_3)_2NCH_2CH_2OH$	Active
Monomethylaminoethanol	$CH_3NHCH_2CH_2OH$	Active
3-Aminopropanol	$NH_2CH_2CH_2CH_2OH$	Inactive
1-Amino-2-propanol	$CH_2NH_2CHOH\ CH_3$	Inactive

Pellet Assay.—In contrast to the foregoing findings, no evidence of anti-inflammatory activity was found when this assay was employed. In these studies the compounds were given orally and parenterally in doses ranging from 0.1 μg to 10 μg/rat. Treatment was started as early as 20 days prior to implanting the pellets and continued until the assay was completed. *N*(2-hydroxyethyl)-palmitamide was also inactive when administered in the diet at a concentration of 1.33 per cent (approximately 200 mg./rat/day) for 8 days before the anti-inflammatory test was initiated. Similarly, it was not possible to demonstrate activity when the phospholipid fraction from egg yolk was fed in the diet at concentrations of 2, 5, and 10 per cent for 21 days, or when ethanolamine was administered subcutaneously at 1 mg./rat for 5 days. Studies with the concentrate from peanut oil, also, showed it to be inactive when administered subcutaneously over a wide range of concentrations.

Evans Blue Assay.—The subcutaneous administration of *N*(2-hydroxyethyl)-palmitamide, ethanolamine, the concentrate from peanut oil, and the oral administration of the palmitamide and the phospholipid fraction from egg yolk failed to influence early stages of inflammation as evaluated by the Evans blue procedure. Attempts to demonstrate a local effect upon capillary permeability to Evans blue were also negative when either *N*(2-hydroxyethyl)-palmitamide or ethanolamine were applied to the cotton pellets before they were implanted in the subcutaneous tissue. In these experiments concentrations of 50, 100, 400, and 1,000 μg of the palmitamide and 400 μg of ethanolamine were employed.

DISCUSSION

Various fractions of soybean lecithin and peanut oil were tested for anti-inflammatory activity in the above bioassays. It shortly became apparent that the active fraction of the natural products had anti-inflammatory activity at low concentrations in the guinea pig. On crystallization of this substance activity was seen at 0.3 μg./Kg. This activity was maximal and no greater decrease in swelling was seen with larger dosage. Similar findings have been reported by Long and Martin in their studies with peanut oil.

The substance, which was found to be the active factor in peanut oil and soybean lecithin, was identified as *N*(2-hydroxyethyl)-palmitamide.* It is possible that other active factors are also present in peanut oil and soybean lecithin. Similarly, *N*(2-hydroxyethyl)-palmitamide was isolated from an alcohol soluble fraction of egg yolk.[6] Such an alcohol soluble fraction of egg yolk was found to be active by Coburn and his associates in their clinical studies, showing the prevention of recrudescence of rheumatic fever in underprivileged children in spite of recurrent attacks of hemolytic streptococcal infection.[11] Although Coburn shows a correlation between activity in the guinea pig assay and clinical efficacy, it is not certain that *N*(2-hydroxyethyl)-palmitamide is the factor responsible for prevention of recurrence of rheumatic fever. Clinical studies with *N*(2-hydroxyethyl)-palmitamide remain to be done in order to clarify this point.

N(2-hydroxyethyl)-palmitamide was synthesized and found to be active at 0.3 μg/Kg. On further degradation, the ethanolamine hydrochloride fragment was found to be active while palmitic acid was inactive. These results led to a search for compounds similar to ethanolamine to correlate chemical structure with biologic activity. As seen from Table III this anti-inflammatory activity has a high degree of specificity. DL-1-Amino-2-propanol and 1-amino-3-propanol were inactive. Furthermore, oxidized compounds, although having an amino group on 2 position such as glycine, serine, sarcosine, and betaine, were inactive.[6] Acetylation of the amino or hydroxyl group of ethanolamine apparently did not change the pharmacologic activity of ethanolamine since *O*-acetylethanolamine hydrochloride, *N*(2-hydroxyethyl)-lauramide, *N*(2-hydroxyethyl)-acetamide, and *N*(2-hydroxyethyl)-salicylamide were active.

Since ethanolamine is converted to choline[12] in the animal body the intermediates of this cycle, monomethylethanolamine and dimethylethanolamine, were assayed for anti-inflammatory activity. Both compounds were active. The significance of the interrelationship between choline and ethanolamine in this biologic phenomenon is difficult to assess and calls for further study.

Except for some preliminary positive findings in the rat formalin assay none of these anti-inflammatory agents were active either in the cotton pellet test (granuloma test) or Evans blue dye test. It should be kept in mind that although all of these assays measure anti-inflammatory activity, the cause of the inflammation and the stage of the inflammatory reaction is different in each assay procedure.

*Dr. D. A. Long has confirmed our work by showing that *N*(2-hydroxyethyl)-palmitamide depressed the sensitivity of BCG infected guinea pigs to tuberculin.[13]

Preliminary studies on mechanism of action of *N*(2-hydroxyethyl)-palmitamide indicate that neither the adrenals nor the hypophysis mediate the observed anti-inflammatory activity.

SUMMARY

The anti-inflammatory activity of various fractions of peanut oil, soybean lecithin, and egg yolk was studied in a modification of Coburn's guinea pig joint anaphylaxis assay. Crystalline fractions were active at 0.3 μg/Kg., and were identified in all 3 cases as *N*(2-hydroxyethyl)-palmitamide.

The synthetic compound was as active as the natural crystalline product. On degradation of this compound ethanolamine was found to be active at 3 μg/Kg. while palmitic acid was inactive. Attempts to substitute various groupings of ethanolamine showed that pharmacologic activity is associated with a high degree of chemical specificity.

These compounds were inactive, however, in the pellet and capillary permeability (Evans blue) tests.

REFERENCES

1. Long, D. A., and Martin, A. J. P.: Factor in Arachis Oil Depressing Sensitivity to Tuberculin in BCG Infected Guinea Pigs, Lancet **1**: 464, 1956.
2. Coburn, A. F., and Moore, L. V.: Nutrition as a Conditioning Factor in the Rheumatic State, Am. J. Dis. Child. **65**: 744, 1943.
3. Coburn, A. F., Graham, C. D., and Haninger, J.: The Effect of Egg Yolk in Diets on Anaphlyactic Arthritis (Passive Arthus Phenomenon) in the Guinea Pig, J. Exper. Med. **100**: 425, 1954.
4. Long, D. A.: The Relationship of the Haemolytic Streptococcus to Rheumatic Fever, Med. Illustrated **10**: 299, 1956.
5. Peete, D. C.: Rheumatic Fever: Diet as a Predisposing Factor, Ann. Int. Med. **21**: 44, 1944.
6. Kuehl, F. A., Jr., Jacob, T. A., Ganley, O. H., Ormond, R. E., and Meisinger, M. A. P.: The Identification of an Anti-Inflammatory Agent Present in Egg Yolk, Soybean Lecithin and Peanut Meal, J. Am. Chem. Soc. **79**: 5577, 1957.
7. Cochran, W., and Cox, G.: Standard Analysis of Variants for Split Plot Designs, Experimental Designs, New York, 1950, John Wiley & Sons, Inc.
8. Dunnett, C. W.: A Multiple Comparison Procedure for Comparing Several Treatments With a Control, J. Am. Stat. Assoc. **50**: 1096, 1955.
9. Meier, R., Schuler, W., and Dessoules, P.: Zur Frage des Mechanismus der Hemmung des Bindegewebswachstums durch Cortisone, Experientia **6**: 469, 1950.
10. Long, D. A.: Personal communications.
11. Coburn, A. F.: Personal communications.
12. Umbreit, W. W.: Metabolic Maps, Minneapolis, 1952, Burgess Publishing Company, p. 263.

A LABORATORY AND CLINICAL EVALUATION OF THE KOLFF COIL KIDNEY

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THE first report of extracorporeal hemodialysis appeared in 1913.[1] Since then, many types of "artificial kidney" have been described, but few are used extensively.[2] Kolff[3] recently described a coil kidney which aroused our interest because of its disposability, compact size, and ease of assemblage. The purported ability to ultrafilter 250 to 300 ml. of water per hour would be useful in patients with excess body water at the time of dialysis.

This report is a laboratory and clinical evaluation of the Kolff coil kidney based on dialysance†[4] across the artificial kidney, on measurements of the degree of red blood cell destruction, and on quantitative aspects of ultrafiltration. Both in vivo and in vitro studies were performed.

METHODS

Details of the Kolff coil kidney are described elsewhere.[3] In essence the apparatus consists of two parallel envelopes of cellulose tubing 10 M. in length coiled around a can 11 cm. in diameter and separated by screens of polyvinyl chloride coated Fiberglas. Inasmuch as there are two cellulose blood compartments, there are necessarily two blood inflow and outflow tubes. The coil‡ is placed in a larger open-top can with a garden hose connection at the bottom for entry of the bath fluid. The arterial blood is forced through the Kolff coil kidney under pressure by a Sigmamotor pump and is returned to the venous cannula after passing through a bubble-clot catcher. The bath fluid is pumped from a 100 L. tank into the unit via the hose connection, between the coils, and overflows back into the tank. This apparatus appears diagrammatically in Fig. 1.

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†Dialysance $= a \frac{A-R}{A-U}$

a = arterial blood flow into artificial kidney in milliliters per minute.
A = arterial or artificial kidney inflow blood concentration of a substance.
R = venous or artificial kidney outflow blood concentration of a substance.
U = bath fluid concentration of a substance.

‡Kindly supplied by the Baxter Laboratories, Inc., Morton Grove, Ill.

The in vitro studies consisted of the dialysance of urea at various blood and bath flow rates in order to evaluate the flow rates for each that would yield the maximal dialysance of urea. Water, saline, and blood, each with added urea, were used to perfuse the coil. When the maximum dialysance of urea was found by varying the "blood" flow rate with a constant bath flow rate, the "blood" flow rate was then kept constant and the flow rate of the bath fluid varied. These results are described below.

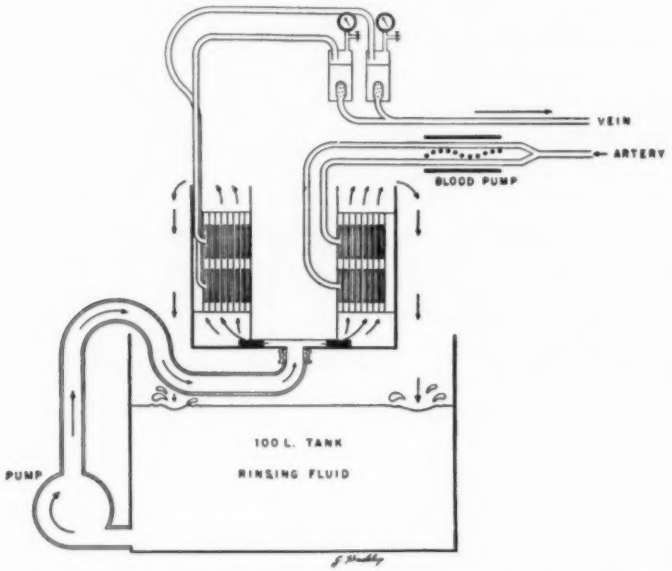

Fig. 1.—Schematic diagram of the Kolff coil unit. (From Kolff and Watschinger: J. Lab. & Clin. Med. 47: 973, 1956.)

The in vivo studies were performed on both dogs and patients, the latter with terminal renal failure. Since it is well established that small animals such as dogs do not tolerate dialysis well because of profound hypotension requiring vasopressor drugs,[6] norarterenol (Levophed) was employed in the dog experiments to maintain blood pressure. The exact mechanism for the hypotension is poorly understood and may be related to minor blood group incompatibilities.[7] Ultrafiltration is generally measured on the basis of change in weight before and after dialysis, but with small animals this was considered unreliable. Other parameters used to assess fluid shift such as hematocrit, osmolarity, and plasma protein concentration are influenced by factors related to dialysis per se and have a high standard error. Small shifts of plasma water, however, may be detected by measuring simultaneously the specific activity of the I^{131} albumin in the inflow and outflow plasma of the Kolff coil kidney. This method has a standard error of less than 2 per cent[10] in our experiments. I^{131} was assayed with a sodium iodide scintillation counter, pulse analyzer, and gamma ray spectrometer.* Urea and uric acid dialysances were performed simultaneously. Urea was measured by the method of Archibald,[5] and uric acid by the method of Praetorius.[9] Plasma hemoglobin was measured by the cyanmethemoglobin method using the Coleman Junior spectrophotometer.

*Manufactured by the Atomic Instrument Company, Cambridge, Mass.

RESULTS

In Vitro Studies.—To determine the maximum dialysance of urea of the Kolff coil kidney as a function of optimal blood flow rate, a water-urea solution was dialyzed against a urea-free water bath. The flow rate of the urea solution was varied and the bath flow rate maintained constant at 4 L. per minute. Fourteen observations with duplicate determinations of the dialysance of urea were carried out at 7 blood flow rates varying from 50 to 450 ml. per minute. The maximum dialysance of urea was observed to be 90 ml. per minute with an extraction ratio of 45 per cent at a blood flow rate of 200 ml. per minute. This compared poorly with similar studies on the Kolff-Brigham rotating kidney where the results were twice those obtained on the Kolff coil kidney.[11] Subsequent data revealed that a bath flow rate of 4 L. per minute was not optimal.

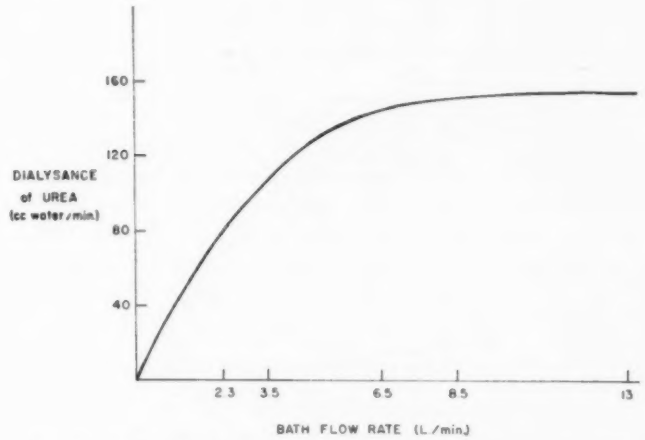

Fig. 2.—Effect of bath flow rate on dialysances of urea. An aqueous solution of urea at a concentration of 100 mg. per cent was perfused through the coil unit at a flow rate of 200 ml. per minute.

The optimal bath flow rate was determined similarly by varying the bath flow rate and maintaining the urea solution flow rate constant in a single experiment at 5 flow rates. Fig. 2 shows that there is a direct relationship between the dialysance of urea and bath flow rate such that the dialysance increases as the bath flow rate increases. The curve begins to plateau at 6 L. per minute although the dialysance shows some increase up to 13.2 L. per minute, the maximum flow rate of the pump.

It was possible that the dialysance of urea in an aqueous solution might differ from that in whole blood since clogging of pores with protein or the adherence to the cellophane of white cells and platelets[14] might progressively interfere with dialysis. Therefore, in a single experiment heparinized whole banked blood with added urea was dialyzed against a urea-free bath having

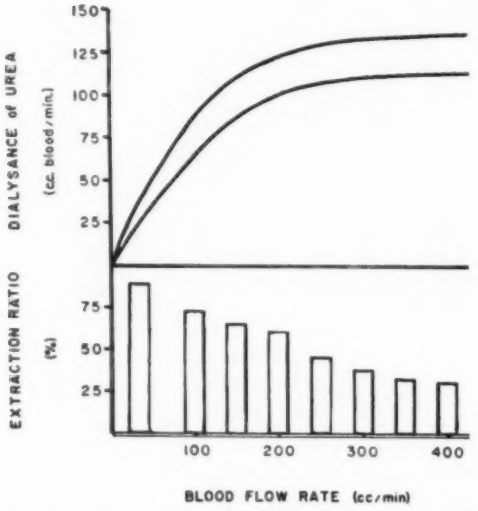

Fig. 3.—In vivo effect of blood flow rate on dialysance of urea and extraction ratio of urea. This data obtained from 7 human dialyses indicates that dialysance of urea increases. The extraction ratio of urea concomitantly falls although the quantity of urea removed increases.

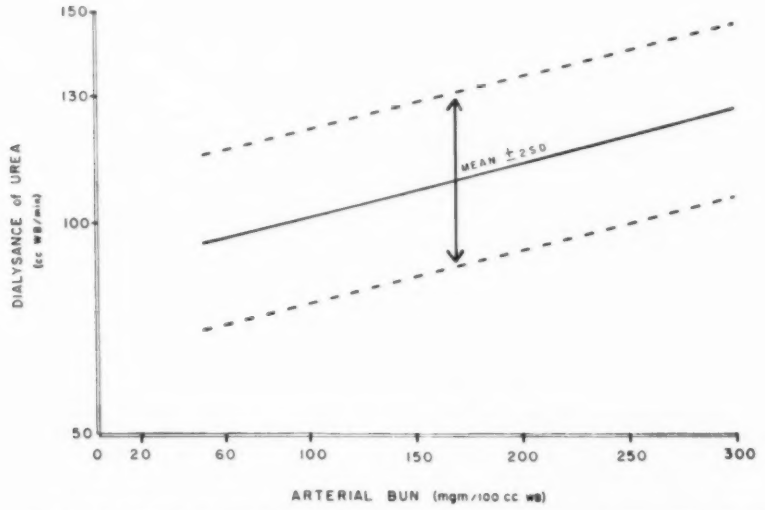

Fig. 4.—Effect of arterial urea concentration on dialysance of urea. The dialysance of urea measured as blood urea nitrogen is a function of the arterial urea concentration and rises as the blood urea increases. In this experiment the bath flow rate was constant at 13 L. per minute.

a composition simulating extracellular fluid. The same unit was used to dialyze a saline solution with added urea both before and after the blood was dialyzed. Dialysances of the urea solution were identical before and after dialysis of the whole blood, suggesting that there was no alteration in the permeability of the cellophane.

In Vivo Studies.—The first dialysis of a dog resulted in marked hemolysis with plasma hemoglobin levels greater than 1 Gm. per cent. This we attributed to mechanical trauma by the Sigmamotor pump. It was noted, however, that all hemolysis was in the heparinized donor blood, i.e., blood used to prime the coil kidney. Since dog erythrocytes are more fragile than human erythrocytes,[8] hemolysis was minimized by aseptically exsanguinating a donor animal by the femoral artery and collecting the blood in acid citrate dextrose (A.C.D.) plastic bags of the type employed in our blood bank. Levophed was administered to the donor dog as blood pressure began to fall, 90 per cent of the animal's theoretical blood volume could be obtained in this way. Dog blood collected and stored at 5° C. in A.C.D. media as described above had preservation characteristics comparable to human blood under similar conditions.[8] Some hemolysis was noted after dialysis employing this dog blood, but plasma hemoglobin levels never exceeded 40 to 50 mg. per cent.

Eight anesthetized dogs weighing 10 to 25 kilograms were selected for initial studies in order to develop our technique in preparation for human use. Urea solution was given by stomach tube to provide initial blood urea nitrogens of 80 to 125 mg. per cent. Ten observations in the 8 animals studied revealed a mean dialysance of urea of 90.2 ml. per minute with a standard deviation of 9.3 ml. per minute.

Seven dialyses were performed on 5 human patients. Five dialyses were undertaken in 3 critically ill uremic patients with chronic renal failure secondary to polycystic disease and 2 others on patients with chronic glomerulonephritis. None of the reported complications of hemodialysis[2] were observed such as pyrogen reactions, hemorrhage, clotting in the apparatus, hemolysis, infection, and a rise in blood pressure during these 7 procedures. The only untoward event was a reversible cardiac arrhythmia, a phenomenon not uncommon during effective hemodialysis.[12] The data obtained from the human dialyses is summarized in Figs. 3, 4, and 5.

Ultrafiltration was evaluated in 2 patients by the intravenous administration of albumin tagged with 5 μc of I^{131}. The specific activity of the plasma from the inflow and outflow tracts of the Kolff coil kidney was assayed, and the amount of water extracted was calculated from this figure and the blood flow rate.

Each of the 14 duplicate determinations carried out demonstrated a higher concentration of I^{131} on the outflow side of the Kolff coil kidney. The change in body weight roughly paralleled the calculated water loss. Ultrafiltration was 200 to 500 ml. per hour. This variation is related to the hydrostatic pressure within the coil, the blood flow rate, and the peripheral resistance in the outflow tract. The osmolarity of blood leaving the Kolff coil kidney was, in

each instance, at least 25 mOsm./L. higher than the bath osmolarity. The inherent resistance of blood flow through the coil results in a hydrostatic pressure of approximately 300 mm. Hg at a blood flow of 200 ml. per minute; this overcomes the osmotic pressure gradient and accounts for the observations cited above. When the resistance is increased on the outflow side of the Kolff coil kidney a further increase in hydrostatic pressure will ensue, resulting in even greater ultrafiltration.

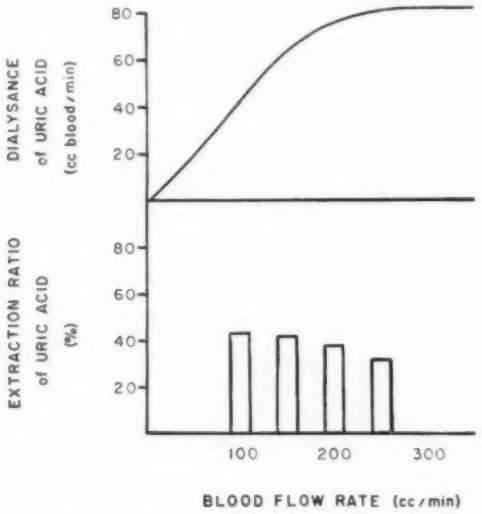

Fig. 5.—In vivo effect of blood flow rate on dialysance of uric acid and extraction ratio of uric acid. Data plotted from twelve determinations during the dialysis of a uremic patient with an initial serum uric acid of 27.8 mg. per cent.

Fifteen plasma hemoglobin determinations were performed during the initial 4 human dialyses. At no time did the plasma hemoglobin exceed 13 mg. per cent above the predialysis level. The average dialysance of uric acid (Fig. 5) was 75 ml. per minute at a blood flow of 200 ml. per minute and bath flow of 13.2 L. per minute. The relative dialysance of uric acid to urea was 0.48 and does not differ significantly from the figure 0.45 cited by Wolf[4] employing the Kolff-Brigham rotating kidney. This ratio is consistent with the relative molecular weights of uric acid and urea and effective diffusion diameters. Eight hours of dialysis resulted in a fall of serum uric acid from 27.8 mg. per cent to 8.8 mg. per cent in the one patient so studied.

DISCUSSION

The dialysance of urea is widely used as a comparative index of efficiency of artificial kidneys. Our urea determinations were done both on whole blood and on plasma. Although plasma urea nitrogen determinations are more reproducible, either method is acceptable since urea is freely diffusible and rapid

equilibrium occurs between the red cell and plasma. The amount of blood or plasma "cleared" is related to the diffusion gradient across the membrane, the rate of blood flow, the concentration of urea in the plasma, and the bath flow rate. Figs. 2 and 3 demonstrate that the dialysance of urea increases with an increase in both bath flow rate and blood flow rate. This rise begins to plateau at a blood flow of 200 ml. per minute, a finding which agrees with the data of Kolff[3] and which is comparable to the Kolff-Brigham rotating kidney.[11] At a blood flow of 200 ml. per minute, the mean dialysance of urea is 110 ml. per minute at a bath flow of 6 to 8 L. per minute. This figure compares favorably with the mean dialysance of urea of 130 ml. per minute obtained with the Kolff-Brigham rotating kidney under comparable clinical conditions. The differences in urea dialysances may be attributed to a difference in cellophane surface area of approximately 15 per cent between the two types of instruments. This fact is somewhat surprising in view of the fact that turbulent flow which favors increased dialysance should be markedly increased by the mechanics of the rotating kidney. Maximum dialysance, however, with either instrument might be achieved by maintaining a blood flow well in excess of the flows employed during these experiments. However, increased flow increases the volume of blood contained within the apparatus[4] and entails some risk of producing hypotension in the patient. The phenomenon of hypotension in the dog during dialysis is probably related in part to the fact that the distensible cellophane will accommodate more blood during the progress of a dialysis particularly at higher flow rates.

In our experiments optimum dialyses on the Kolff coil kidney averaged approximately 110 ml. per minute or significantly less than 140 ml. per minute quoted by Kolff.[3, 13] As in other types of artificial kidneys extraction ratios are inversely related to flow rate although the total amount of urea removed is directly related to flow; as is true for other instruments there is a linear increase of dialysance (Fig. 4) which occurs as a function of increasing concentrations of blood urea nitrogen.

Uric acid dialysances are rarely measured and infrequently used as a determinant of artificial kidney efficiency. A comparison of Fig. 3 and 5 indicates, however, a similarity in the slope of dialysances between urea and uric acid.

The ability to remove water from the blood by ultrafiltration is an asset of the coil kidney not shared by the rotating model. In the former, blood is pumped through the cellophane coils under a pressure of 180 to 300 mm. Hg. This hydrostatic gradient results in the ultrafiltration of 200 to 500 ml. of water per hour. The ability to remove water may be a decided advantage during the dialysis of the overhydrated uremic patient.

In spite of mechanical trauma to red blood cells hemolysis was not clinically significant.

The Kolff coil kidney including all the connecting tubing is supplied sterile and can be quickly assembled. The coils themselves are disposable units

obviating the need for special personnel to clean and reassemble them following each dialysis. The initial cost of the total unit including tank and pumps is relatively low when compared to the Kolff-Brigham model. Partly offsetting this advantage, however, is the expense of the disposable units each of which can be only used once. Furthermore a disadvantage peculiar to this model is the fact that a leak in the cellophane necessitates the discarding of the entire cellophane unit along with the contained blood.

The Kolff coil kidney has the same major disadvantages of the rotating model; namely, it is not portable, it is not financially self-supporting, and it requires highly skilled professional personnel in an institution whose laboratory, medical, surgical, and nursing facilities are adequate for the routine and emergency demands that arise. As in any other type of efficient hemodialyzer profound changes in the composition of extracellular fluid may be effected and its use demands of the operator more than a passing knowledge of the physiology of body fluids and the consequences of marked derangements therein. Therefore, in spite of its efficacy and convenience, this apparatus, as are others of its type, is safe and effective only in relation to the skill and experience of its operator.

SUMMARY AND CONCLUSIONS

1. In vitro dialysances of urea solutions indicate that the Kolff coil kidney per unit area of cellophane employed compares favorably with other types of artificial kidneys.

2. The Kolff coil kidney has the additional advantages of low initial expense, disposability of parts requiring sterilization, and the ability to remove water by ultrafiltration.

3. Mechanically it is a safe and efficient apparatus for clinical hemodialysis but depends as do instruments of other design upon the skill and experience of its operators for effective and safe clinical hemodialysis.

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REFERENCES

1. Abel, J. J., Rountree, L. G., and Turner, B. B.: The Removal of Diffusable Substances From the Blood by Means of Dialysis, Tr. A. Am. Physicians **28:** 51-54, 1913.
2. Merrill, J. P.: Medical Progress: The Artificial Kidney, New England J. Med. **246:** 17-27, 1952.
3. Kolff, W. J., and Watschinger, B.: Further Development of a Coil Kidney, J. LAB. & CLIN. MED. **47:** 969-977, 1956.
4. Wolf, A. V., Remp, D. G., Kiley, J. E., and Currie, G. D.: Artificial Kidney Function: Kinetics of Hemodialysis, J. Clin. Invest. **30:** 1062-1070, 1951.
5. Archibald, R. M.: Colorimetric Determination of Urea, J. Biol. Chem. **157:** 507-518, 1945.
6. Maher, F. T., Young, J. V., Watkins, L. E., and Bollman, J. L.: The Laboratory Experiences With the Skeggs-Leonards Artificial Kidney, Proc. Staff Meet., Mayo Clin. **31:** 350-358, 1956.
7. Maher, F. T., and Edwards, E. E.: The Repeated Extracorporeal Hemodialysis of Trained Normal Dogs. Paper Presented at Meeting of American Society for Artificial Internal Organs, Chicago, Ill., April 14, 1957.
8. Swisher, Scott N.: Personal communication.

9. Praetorius, E.: An Engymatic Method for the Determination of Uric Acid by Ultraviolet Spectrophotometry, Scandinav. J. Clin. Lab. Invest. **1**: 222-230, 1949.
10. Gibson, J. G., II: Personal communication.
11. Merrill, J. P., Thorn, G. W., Walter, C. W., Callahan, E. J., III, and Smith, L. H., Jr.: The Use of the Artificial Kidney. I. Technique, J. Clin. Invest. **29**: 412-424, 1950.
12. Merrill, J. P.: The Use of the Artificial Kidney in the Treatment of Glomerulonephritis, J. Chron. Dis. **5**: 138-152, 1957.
13. Kolff, W. J., Watschinger, B., and Vertes, V.: Results in Patients Treated With the Coil Kidney (Disposable Dialyzing Unit), J. A. M. A. **161**: 1433-1437, 1956.
14. de Leeuw, N. K. M., and Blaustein, A.: Studies of Blood Passed Through Artificial Kidney, Blood **4**: 653-666, 1949.

THE EFFECT OF PREDNISOLONE ON CIRCULATING ANTIBODY FORMATION IN ANIMALS IMMUNIZED WITH HUMAN PLATELET ANTIGEN

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THE adrenal cortical hormones have been shown to inhibit antibody response to certain antigens.[1-6] The influence of ACTH and cortisone on antibody formation has been variable and appears to be dependent upon the nature of the antigenic stimulus, the animal employed, the method of immunization, and the amount of these two hormones administered. Circulating antibodies or agglutinins to platelets have been described in certain thrombocytopenic states.[7-10] It is conceivable, therefore, that the beneficial effect of cortisone, when it does occur, may be related to a suppression of the level of circulating antibodies to platelets. The difficulty in detecting antibodies to platelets in human sera is well recognized. It is generally accepted that the present techniques of studying the immune characteristics of platelets lack consistency and specificity. Hence, a more sensitive and specific method for determining the presence of antibodies to platelets is desirable.

A method for demonstrating antibodies utilizing bis-diazo-benzidine to conjugate antigen to erythrocytes has been described by Stavitsky and Arquilla.[11] These investigators demonstrated that this hemagglutination method was sensitive and specific for the detection of insulin antibodies.[12, 13] Accordingly, the present investigation was undertaken to determine whether this technique would be applicable to the study of the immune characteristics of platelets. It was found that rabbit antiserum to platelets could be detected by this method. Furthermore, prednisolone (Δ^1-hydrocortisone) was observed to inhibit the formation of circulating antibodies to human platelets in the rabbit.

METHODS AND MATERIALS

Antigen Preparations.—Human platelet antigen was prepared from 6 individuals with normal platelets, morphologically and functionally, in so far as blood coagulation and clot retraction studies were concerned. Two hundred and fifty milliliters of blood was obtained from each individual, using 5 per cent sequestrene as anticoagulant. Siliconized glassware

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and coated needles were used for the collection and separation of the platelets. The platelets were separated by differential centrifugation and washed 3 times with normal saline. Platelets so collected always contained small numbers of erythrocytes. The packed platelets were then frozen and stored at -20° C. until used. The platelets obtained from each individual were thawed and 5 ml. of normal saline was added. This suspension was homogenized in a ground glass tissue grinder and pooled with similarly treated platelets from other individuals. An aliquot of pooled platelet homogenate was incorporated into an equal volume of adjuvant (falba 1 part, mineral oil 2 parts).

In order to avoid nonspecific agglutination, the platelet antigen employed in sensitizing erythrocytes for the hemagglutination test was prepared as follows. Pooled platelet homogeneate was diluted with an equal volume of normal saline and centrifuged at 4,000 r.p.m. for 30 minutes. The supernatant was removed and was adjusted so that 1.0 ml. contained approximately 1.5 to 2.0 mg. of protein, as determined by micro-Keldjahl analysis. This was stored at -20° C. and constituted the soluble protein fraction for conjugation to the erythrocyte.

Solutions.—Bis-diazo-benzidine (BDB) was prepared according to the method of Kabat and Mayer[14] and stored at -60° C.

The diluent was 1:100 normal rabbit serum (NRS) in veronal buffered saline at pH 7.45.[14] The NRS was inactivated at 56° C. for 30 minutes and absorbed with an equal volume of human AB+ and O+ erythrocytes at 37° C. for 30 minutes.

Prednisolone* was prepared by adding the appropriate volume of sterile saline to the dry powder so that the final concentration was 1.5 mg. of prednisolone per millilter. This solution was prepared daily.

Erythrocyte Preparation.—The AB+ and O+ cells used for absorption were obtained from the blood bank and washed at least 3 times with normal saline. The O negative cells to which the platelet antigen was conjugated were collected in sterile Alsever's solution[14] and stored at 3 to 4° C. Cells were not used for hemagglutination which had been stored for more than 2 weeks.

The O negative erythrocytes were sensitized with the soluble platelet protein fraction by the method described by Stavitsky and Arquilla.[11] It was necessary to determine the optimal ratio of BDB to the soluble platelet antigen for the sensitization of the erythrocytes. The ratio was 1.6 to 2.0 mg. of platelet protein to 0.5 ml. of 1:15 dilution of BDB. Centrifugation of the soluble platelet antigen at 60,000 g in an ultracentrifuge for 30 minutes did not alter the ratio.

Immunization.—Eight white rabbits weighing 2.5 to 3.0 kilograms were immunized by injecting 1.0 ml. of the platelet antigen-adjuvant mixture subcutaneously in 8 different sites along the back. Control samples of blood were obtained from each animal prior to injection.

The effect of prednisolone on antibody formation was studied as follows. Four rabbits served as controls and the remaining 4 rabbits were treated with 3.0 mg. of prednisolone daily by subcutaneous injection in 2 divided doses for 6 weeks. At the end of the fifth week all animals received a booster dose of the pooled platelet antigen containing 5.0 mg. of protein. A second booster was given at the end of the seventh week. This enabled observations to be made on whether the level of circulating antibodies would rise during the week that prednisolone was discontinued. Except for the first week, blood was obtained at weekly intervals. All of the collected sera were inactived at 56° C., absorbed with an equal volume of washed packed human AB+ and O+ cells and stored at -20° C. until tested. Each animal received a total dose of 100 mg. of protein antigen as determined by micro-Keldjahl analysis. The serum antiplatelet titer of the rabbits receiving prednisolone was compared with the untreated control animals.

Detection of platelet antibodies by hemagglutination.—Two series of doubling dilutions of each test antiserum ranging from 1:5 to 1:2,500 were prepared in 10 by 75 mm. test tubes with 1:100 NRS in veronal buffered saline as the diluent in a final volume of 0.5 ml. To one series of doubling dilutions 20 μg of soluble platelet antigen protein was added in 0.1 ml. To

*Supplied by The Upjohn Company, Kalamazoo, Mich.

the second series, 0.1 ml. of diluent was added. After mixing, 0.05 ml. of a 2 per cent suspension of sensitized erythrocytes was added to each tube making a final volume of 0.65 ml. per tube. Controls, consisting of unsensitized erythrocytes in 1:5 dilution of antiserum and sensitized erythrocytes in 1:5 dilution of normal rabbit serum, were run in duplicate at all times. Tubes were shaken and allowed to stand for 3 to 4 hours. Agglutination was readily determined by the pattern of erythrocytes at the bottom of the tube. The criteria for the presence of antibodies to platelets in any given serum were established as follows:

1. The agglutination by the serum of erythrocytes conjugated with soluble platelet antigen.
2. The inhibition of this agglutination when the serum was mixed with the soluble platelet antigen prior to the addition of the sensitized erythrocytes.
3. The absence of agglutination when the serum was mixed with nonsensitized cells.
4. The absence of spontaneous agglutination of the sensitized erythrocytes when suspended in diluent of normal rabbit serum.

The titer of the antiserum was designated as the highest dilution in which there was definite evidence of agglutination.

Specificity.—The rabbit antiplatelet serum was found to have a high degree of specificity for the agglutination of erythrocytes sensitized with a soluble platelet protein. Cross reaction between rabbit antileukocytic serum and erythrocytes conjugated with platelet protein was not observed. Conversely, rabbit antiplatelet serum did not cause agglutination of erythrocytes sensitized with a soluble protein fraction obtained from human leukocytes. Furthermore, the leukocytic protein fraction did not inhibit the hemagglutination reaction when mixed with the antiplatelet serum prior to the addition of erythrocytes sensitized with the platelet protein fraction. Similarly, other proteins, including fibrinogen, albumin, and normal human sera, failed to neutralize the rabbit antiplatelet antiserum. A potent Coombs' serum did not agglutinate sensitized cells nor inhibit the hemagglutination reaction.

It appeared that the rabbit antiplatelet serum does not react to any demonstrable degree with soluble leukocytic fraction or substances present in normal serum. It also appears that the soluble platelet antigen is immunologically distinct from the leukocyte or serum fractions employed. (Table I.)

TABLE I. SPECIFICITY OF HEMAGGLUTINATION REACTION

RABBIT SERA	SERUM TITER SENSITIZED ERYTHROCYTES	
	WITH PLATELET ANTIGEN	WITH LEUKOCYTE ANTIGEN
Antiplatelet sera	1:1280	0
Antileukocyte sera	0	1:640
Antiglobulin sera	0	0
Other antisera		
Anti A, B, D, M, and N	0	0

TABLE II. INITIAL RESPONSE DURING IMMUNIZATION

ANIMAL	TREATMENT	TITER	
		4 WEEKS	5 WEEKS
1	None	1:20	1:40
2	None	1:40	1:80
3	None	1:40	1:160
4	None	1:40	1:80
5	Prednisolone*	0	1:5
6	Prednisolone*	0	0
7	Prednisolone*	0	0
8	Prednisolone*	0	1:5

*Prednisolone 3.0 mg. daily given subcutaneously.

RESULTS

In the 4 control animals, the antiserum titer rose gradually and at the end of 5 weeks following the initial injection of antigen the titer ranged from 1:40 to 1:160 (Table II). In contrast to this, the animals treated with prednisolone did not develop any detectable antibodies by the fourth week, and after 5 weeks, only 2 animals had a measurable titer of 1:5.

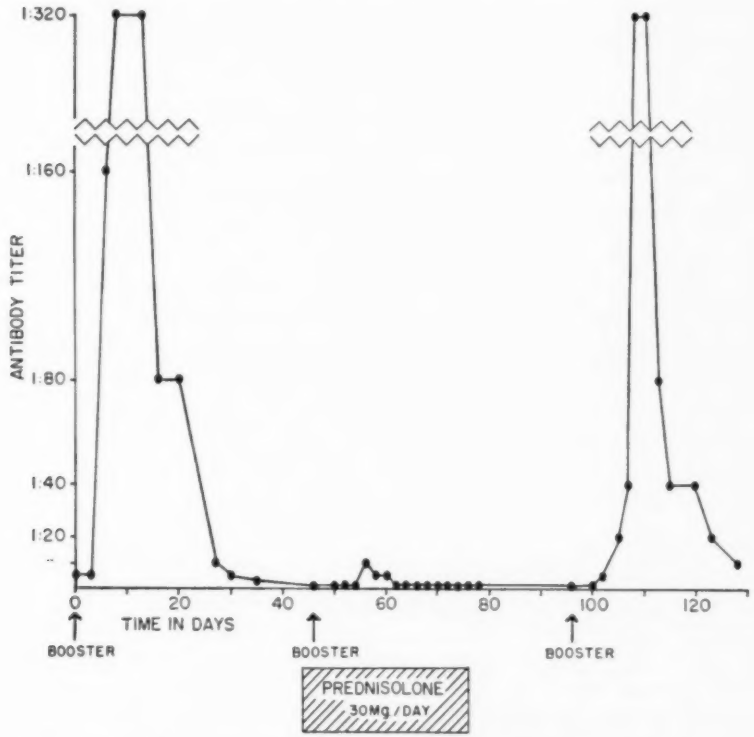

Fig. 1.—The influence of prednisolone on the anamnestic response to human platelet antigen. This figure demonstrates the marked depression of circulating antibodies in a rabbit treated with 30 mg. of prednisolone daily. A subsequent booster dose of antigen produces a characteristic anamnestic response.

When a booster injection was administered the control animals showed a characteristic anamnestic response. Seven days following the booster injection the antiplatelet titer of the controls was 1:160 to 1:640, whereas the antiplatelet titer of the prednisolone-treated animals was only 1:40 to 1:80 7 days following an identical booster injection (Table III). Prednisolone was then discontinued and the antibody titers determined after one week. At this time there was no demonstrable rise in the level of antibodies of soluble platelet antigen in either group of animals.

A second booster injection was administered 14 days after the initial booster. Seven days later the control animals showed little change in circulating antibody with the exception of one animal whose titer rose from 1:320 to 1:1,280. In contrast to this, all but one of the previously treated animals responded with a definite increase in antibody titers ranging from 1:80 to 1:320 (Table III).

TABLE III. ANAMNESTIC RESPONSE

ANIMAL	TREATMENT	SERUM TITER		
		7 DAYS AFTER FIRST BOOSTER	14 DAYS AFTER* FIRST BOOSTER	7 DAYS AFTER† SECOND BOOSTER
1	None	1:320	1:320	1:320
2	None	1:160	1:320	1:320
3	None	1:320	1:320	1:1,280
4	None	1:640	1:640	1:640
5	Prednisolone 3.0 mg. daily	1:40	1:80	1:320
6	Prednisolone 3.0 mg. daily	1:80	1:40	1:80
7	Prednisolone 3.0 mg. daily	1:40	1:40	1:40
8	Prednisolone 3.0 mg. daily	1:40	1:20	1:160

*No prednisolone for 7 days.
†No prednisolone for 14 days. Interval between first and second booster, 14 days.

In addition, when massive doses of prednisolone, 30 mg. daily, were administered there was almost complete inhibition of the formation of detectable antibody to soluble platelet antigen. However, when the drug was discontinued the anamnestic response did not seem to be impaired, in spite of previous massive amounts of prednisolone (Fig. 1).

DISCUSSION

The results of detecting platelet agglutinins using the whole platelet method described by Harrington[15] and Stefanini[16] has been difficult to repeat in other laboratories. One of the disadvantages of this method has been the occurrence of spontaneous clumping of platelets due to the inherent stickiness of the platelet's surface. Therefore, the detection of platelet agglutinins in test sera has been difficult to appraise objectively. Kissmeyer-Nielsen[17] demonstrated rabbit antibodies against human platelets by hemagglutination of antigen coated erythrocytes previously treated with tannic acid. This method proved to be more sensitive than the direct platelet technique, but when human sera was tested for the presence of platelet antibodies, some normal sera gave positive results. A complement fixation method for the detection of antibodies in rabbits against guinea pig platelets has been described by de Nicola and co-workers.[18] However, complement fixation was not obtained when the sera of patients with idiopathic thrombocytopenic purpura were tested against normal human platelet antigen. The failure to find platelet agglutinins in some cases of thrombocytopenic purpura may be due to lack of an immune mechanism or to an excess of circulating platelet antigen with subsequent neutralization of circulating antibody. In fact, it is surprising that any agglutinins can be demonstrated in the presence of circulating platelets.

The method of hemagglutination used in this investigation for the detection of rabbit antibodies to human platelets is consistent and reproducible. The antigen conjugated to the erythrocyte obviously contained many components, but was immunologically distinct from leukocyte extracts, normal serum, and the A, B, D, M, and N blood group substances.

It appears that this method has a degree of specificity which was not demonstrated by the previously described techniques of other investigators. Therefore, the hemagglutination of erythrocytes sensitized with soluble platelet protein by bis-diazo-benzidine as an indicator for platelet antibodies may have useful clinical application. In addition the inhibition of hemagglutination by platelet antigen may be useful for the detection of circulating platelet protein in thrombocytopenic subjects. One can speculate that the measurement of circulating soluble platelet protein in idiopathic thrombocytopenic purpura and normals may reflect the relative rates of platelet destruction. It is possible that such a determination may have useful prognostic value in determining the course of the disease. On the other hand, the soluble platelet protein employed in these experiments, may have no relationship to the disease state in humans.

The observation that in vitro prednisolone does not inhibit the agglutination of erythrocytes sensitized with soluble platelet protein by rabbit antiplatelet serum raises the possibility that it may not inhibit the antibody from combining with platelets in the intact animal. It has been observed that massive doses of prednisolone elicits a rise in circulating platelets in certain thrombocytopenic states when ordinary doses have been ineffective.[19] The mechanism of this response is unknown. However, it is possible that the massive doses of prednisolone suppress the formation of autoantibodies to platelets. The results observed in the rabbit support this hypothesis. Thus prednisolone inhibited the formation of circulating antibodies to human platelets in rabbits. However, a sensitized state was produced in spite of continued administration of prednisolone. This is also consistent with the clinical observation that thrombocytopenia recurs when prednisolone is discontinued in patients who previously responded to massive doses.

SUMMARY

Detection of human platelet antibodies in the rabbit by the hemagglutination of erythrocytes conjugated with a soluble platelet protein in the presence of bis-diazo-benzidine is described. The soluble platelet fraction employed as the antigen appeared to be immunologically distinct from leukocyte extracts, serum components, and blood group substances.

Prednisolone inhibits the formation of circulating antibodies to human platelets in the rabbit, but does not prevent the production of a sensitized state in the rabbit against human platelet antigen. Massive doses of prednisolone do not permanently alter the rabbit's capacity to react to subsequent booster injections of antigen.

REFERENCES

1. Bjorneboe, M., Fischel, E. E., and Stoerk, H. C.: Effect of Cortisone and ACTH on Concentration of Circulating Antibody, J. Exper. Med. 93: 37, 1951.

2. Germuth, F., Oyama, J., and Ottinger, B.: Mechanism of Action of Cortisone and ACTH in Experimental Hypersensitivity in Rabbits, J. Exper. Med. **94**: 139, 1951.
3. Halpern, B. N., Mauric, G., Holzer, A., and Briot, M.: Effect of Cortisone Upon Rise of Serum Antibody Level After Injection of a Saturation Dose of Antigen in Rabbits Sensitized to Ovalbumin, J. Allergy **23**: 303, 1952.
4. Overman, J. R., and Hanan, R.: Effect of Cortisone on Mumps Antibody Formation, Proc. Soc. Exper. Biol. & Med. **82**: 427, 1953.
5. Hanan, R., and Overban, J. R.: Cortisone Effect on Antibody Levels in Rabbits Simultaneously Immunized With Serum Albumin and Sheep Cells, Proc. Soc. Exper. Biol. & Med. **84**: 420, 1953.
6. Solotorovsky, M., and Winsten, S.: Inhibition of Fatal Anaphylactic Shock in Mouse With Cortisone, J. Immunol. **72**: 177, 1954.
7. Evans, R. S., Takahashi, K., Duane, R. T., Payne, R., and Liu, C.: Primary Thrombocytopenic Purpura and Acquired Hemolytic Anemia, A. M. A. Arch. Int. Med. **87**: 48, 1951.
8. Harrington, W. J., Sprague, C. C., Minnich, V., Moore, C. V., Ahlvin, R. C., and Dubach, R.: Immunologic Mechanisms in Idiopathic and Neonatal Thrombocytopenic Purpura, Ann. Int. Med. **38**: 433, 1953.
9. Tullis, J. L.: Platelet Antibody Tests in the Diagnosis of Purpura, New England J. Med. **249**: 591, 1953.
10. Ackroyd, J. F.: Platelet Agglutinins and Lysins in Thrombocytopenic Purpura, Brit. M. Bull. **11**: 28, 1955.
11. Stavitsky, A. B., and Arquilla, E. R.: Micromethods for the Study of Proteins and Antibodies. III. Procedure and Applications of Hemagglutination and Hemagglutination-Inhibition Reactions With Bis-Diazotized Benzidine and Protein Conjugated Red Blood Cells, J. Immunol. **74**: 306, 1955.
12. Arquilla, E. R., and Stavitsky, A. B.: The Production and Identification of Antibodies to Insulin and Their Use in Assaying Insulin, J. Clin. Invest. **35**: 458, 1956.
13. Arquilla, E. R., and Stavitsky, A. B.: Evidence for the Insulin Directed Specificity of Rabbit Anti-Insulin Serum, J. Clin. Invest. **35**: 467, 1956.
14. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Ill., 1948, Charles C Thomas, Publisher.
15. Harrington, W. J.: Clinical Significance of Antibodies for Platelets, Sang **25**: 712, 1954.
16. Stefanini, M., Plitman, G. I., Dameshek, W., Chatterjea, J. B., and Mednicoff, I. B.: Studies on Platelets. XI. Antigenicity of Platelets and Evidence of Platelet Groups and Types in Man, J. Lab. & Clin. Med. **42**: 723, 1953.
17. Kissmeyer-Nielsen, F.: Demonstration of Platelet Antibodies by Hemagglutination of Antigen-Coated Tanned Erythrocytes, Sang **26**: 117, 1955.
18. de Nicola, P., Rosti, P., and Zangaglia, O.: Complement Fixation Test Due to the Interaction of Specific Antiplatelet Serum and Heterologous Platelet Antigen, J. Lab. & Clin. Med. **45**: 725, 1955.
19. Weisberger, A. S., Suhrland, L., and Arquilla, E.: Massive Steroid Therapy in Resistant Thrombocytopenia, J. Lab. & Clin. Med. (Abst.) **48**: 957, 1956.

INHIBITION OF POLYMORPHONUCLEAR PHAGOCYTOSIS BY ANTIBODIES AGAINST WHITE BLOOD CELLS

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THE ability of specific opsonins to stimulate phagocytosis has been recognized for many years.[1, 2] The possibility that an immunologic mechanism directed against the phagocyte may alter its function has been suggested by more recent investigations. Hickie[3] demonstrated a decrease of phagocytic capacity of normal leukocytes when treated with the serum of a patient with chronic agranulocytosis. Dausset, Nenna, and Brecy[4] reported that the exposure of normal leukocytes to serum obtained from some cases of leukopenia produced leukoagglutination. The remaining free leukocytes appeared to exhibit a greater phagocytic capacity than did the clumped cells. Delaunay, Pages, and Maurin[5] prepared an antiguinea pig leukocyte serum, which appeared to depress the normal attractions of guinea pig leukocytes to starch granules. Merchant and Chamberlain[6] incubated filtrates from cultures of gram-positive cocci, their respective guinea pig antisera and normal guinea pig leukocytes. On subsequent reincubation of this system with heat-killed *Bacillus anthracis*, phagocytosis was inhibited.

In view of the above findings, which suggest a relationship between immunologic factors and inhibition of leukocyte activity, the purpose of the present study is to observe, by in vitro methods, the phagocytic behavior of human polymorphonuclear leukocytes in the presence of antibodies against leukocytes.

METHODS AND MATERIALS

*Procedure for Obtaining Antihuman Leukocyte Rabbit Serum.**—Thirty milliliters of blood was aspirated from a normal donor, under sterile conditions, into a 50 ml. syringe containing 0.9 ml. of 5 per cent solution of ethylenediaminetetraacetic acid and 4.5 ml. of 6 per cent solution of dextran (fraction with intrinsic viscosity 0.40). The technique of Klein, Eridani, Djerassi, and Resnick[8] was employed for the separation of leukocytes from whole blood.

All glass and metal surfaces in contact with white blood cells were rendered nonwettable by treatment with Silicone,† and Arquad,‡ respectively.[7]

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*In the present study, the terms "serum" or "antiserum" have been employed to designate the respective plasma in view of current immunologic terminology.

†General Electric Co., Silicone No. 9977.

‡Armour & Company, Chicago, Ill. No. 75, Lot 701.

After sedimentation of the red blood cells in the syringe, the supernatant plasma-white blood cell suspension was centrifuged at 650 g for 10 minutes. The leukocyte sediment in each centrifuge tube was resuspended in 5 ml. of sterile buffer (consisting of 10 ml. of a stock buffer* and 90 ml. of distilled water). The pH was adjusted to 7.5 with a 4 per cent $NaHCO_3$.

The suspension of leukocytes in buffer was centrifuged at 650 g for 7 minutes. Washing was continued until 10 drops of a 30 per cent solution of trichloroacetic acid failed to produce turbidity in the supernatant; usually 5 washings were required for this purpose. The washed leukocyte sediment was resuspended in buffer.

Phase contrast observations (magnification ×970) indicated morphologic integrity of the white blood cells. The leukocyte suspension contained, on the average, 70 to 80 per cent granulocytes, 15 to 25 per cent lymphocytes, and 5 to 10 per cent monocytes. The total white blood cell count ranged from 6 to 15 million leukocytes per milliliter. By aseptic technique, 1 ml. of leukocyte suspension per injection was given intraperitoneally to each rabbit for a series of 8 biweekly injections. An interval of several months was allowed to elapse before a booster dose of 1 ml. of suspension containing 150 million W.B.C. per milliliter was administered. One week following the administration of the booster dose, the animal was exsanguinated by intracardiac puncture.

The blood was rendered incoagulable by the addition of EDTA (0.6 ml. of a 5 per cent solution of disodium sequestrene per 10 ml. of blood). The serum was separated by centrifugation and stored at −15° C. Serum from nonimmunized rabbits was similarly treated to serve as control.

Procedure for Testing the Effect of Antihuman Leukocyte Serum on Phagocytosis.— This procedure is a modification of the method of Minor and Burnett.[9]

Materials.—

1. *Leukocytes:* Leukocyte suspensions were prepared[8] from 10 ml. samples of blood obtained from presumably normal individuals.

2. *Suspension of starch granules:* Thirty milligrams of starch granules (*Amaranthus cruentis*)† were suspended in 1 ml. of buffer. For use as phagocytic substrate, the suspension was diluted fifty fold with buffer. The diluted suspension was used for 24 hours only.

3. *Magnesium acetate:* A 0.03 M solution in buffer was employed to increase phagocytic activity.[10]

4. *Serum:* Antileukocytic serum, normal rabbit serum (described above), and normal human serum, respectively.

5. *Stock buffer:* This was diluted tenfold as above.

Silicone-treated 3 ml. test tubes were used as incubation vessels. The incubation mixtures were prepared as follows‡: to 0.1 ml. of serum (antileukocyte rabbit, normal rabbit, and normal human, respectively) were added 0.2 ml. of starch substrate, 0.1 ml. leukocyte suspension, and 0.05 ml. of 0.03 M magnesium acetate solution. The white blood cell suspension was added last.

These mixtures were incubated at 37° C. for 20 minutes. A sample of the mixture was then examined under phase contrast (magnification ×970). Phagocytic activity was estimated by determining the number of cells per 100 neutrophils which contained one or more starch granules.

RESULTS

The phagocytic activity of the neutrophils of 19 normal donors was studied. The data are shown in Table I.‡ It is apparent that phagocytosis of starch

*The stock buffer contained 1.2 Gm. $Na_2HPO_4 \cdot 2\ H_2O$, 1.2 Gm. KH_2PO_4, 75 Gm. NaCl, and 8 Gm. KCl per 1,000 c.c. of distilled water.

†Gratefully received from Dr. L. Burnett, Department of Experimental Cytology, Lenox Hill Hospital, New York, N. Y.

‡The human serum has been used as control to determine the capacity of the donor leukocytes to phagocytose in the absence of foreign rabbit protein. This control is not essential when an adequate phagocytic activity is obtained in the control rabbit. In a number of cases, therefore, phagocytosis in the human control was not determined.

granules by polymorphonuclear leukocytes is markedly decreased in the presence of antileukocyte antibodies.

TABLE I. PER CENT PHAGOCYTIC NEUTROPHILS

WHITE CELL SPECIMEN NO.	ANTILEUKOCYTIC RABBIT SERUM	NORMAL RABBIT SERUM	NORMAL HUMAN SERUM
201—A. L.	13	90	—
202—O. M.	2	50	—
203—B. C.	1	45	—
204—D. L.	0	25	68
205—R. F.	1	50	—
206—D. M.	4	62	40
207—N. S.	6	80 to 100	75
208—V. R.	10	74	—
209—A. F.	1	82	62
210—C. L.	6	93	86
211—B. N.	3	78	98
212—G. A.	4	100	79
213—O. B.	11	71	91
214—G. Y.	1	87	77
215—N. S.	0	87	88
216—S. V.	5	70	95
217—G. R.	3	75	—
218—I. N.	14	65	—
219—M. E.	5	66	—

DISCUSSION

The incubation of normal human polymorphonuclear leukocytes with an antihuman leukocyte rabbit plasma markedly depressed the phagocytosis of starch granules. Direct cell counts indicate that the number of leukocytes does not decrease after they have been exposed to antibodies for 24 hours. This would appear to eliminate lytic activity on a large scale, but does not exclude the possibility that phagocytosis has been inhibited by the products of leukocytolysis of a small number of cells.

Additional experiments indicate that the mechanism of the inhibition of phagocytosis is distinct from permanent cell damage since considerable reversibility of the inhibitory effect has been obtained. Whether the partially reversible nature of the process is a manifestation of the instability of the antibody, or whether the neutrophilic leukocyte in the course of prolonged incubation phagocytoses or pinocytoses the antibody is not known.

In serial observations, egestion of the phagocytosed particles by polymorphonuclear leukocytes[11] in the presence of specific antiserum has not been observed. The events described, therefore, appear to reflect a direct, primary action of the antibodies on the polymorphonuclear leukocyte.

Further experiments show that there is less inhibition of phagocytosis as the concentration of antiserum decreases. Agglutination of white cells, which is minimal at higher concentrations of antiserum, becomes more pronounced as the titer of antibodies is decreased. Maximal agglutination occurs at titers which are too low for effecting inhibition of phagocytosis. This demonstration of the prozone phenomenon for human leukocyte agglutination is analogous to the observation of Moeschlin, Meyer, Israels, and Tarr-Gloor[12] on the agglutination of guinea pig leukocytes by specific antiserum. It is of interest

that two qualitatively different phenomena, i.e., the inhibition of phagocytosis and the agglutination of white cells, occur at different concentrations of the antiserum.

A markedly increased susceptibility to disseminated infection in patients with agranulocytosis is well known.[13-17] This has been attributed to the reduced number of circulating phagocytic cells. On the basis of this study, it appears that inhibition of phagocytosis may be an additional factor which predisposes toward sepsis in those cases of agranulocytosis in which antibodies to leukocytes occur.[18-22] A definite relation between phagocytosis and resistance to infection, however, has not been established.[23] Since it appears that there may be an inverse relation between the concentration of antibodies required for agglutination and for the inhibition of phagocytosis, respectively, and since only agglutination would cause a decrease in the number of polymorphonuclear leukocytes, it is feasible that inhibition of phagocytosis might occur without granulocytopenia.

SUMMARY

Antibodies against human white blood cells produced in the rabbit have been shown to inhibit phagocytosis by human polymorphonuclear leukocytes.

REFERENCES

1. Mennes, F.: Das Antipneumokken-Serum und der Mekaniswies der Immunitat des Kaninchens gagen des Pneumo-kokkus, Ztschr. Hyg. **25**: 413, 1897.
2. Marchard, F.: Cited by Topley and Wilson. Principles of Bacteriology and Immunity, New York, 1929, William Woods & Co., p. 173.
3. Hickie, B.: Experimental Observations on Chronic Agranulocytosis, Quart. J. Med. **17**: 165, 1948.
4. Dausset, J., Nenna, A., and Brecy, H.: Leuko-agglutinins. V. Leuko-agglutinins in Chronic Idiopathic or Symptomatic Pancytopenia and in Paroxysmal Nocturnal Hemoglobinuria, Blood **9**: 696, 1954.
5. Delaunay, H., Pages, J., and Maurin, M.: Étude d'un Serum Antileukocytaine, Ann. Inst. Pasteur **73**: 7, 1947.
6. Merchant, D. J., and Chamberlain, R. E.: A Phagocytosis Inhibition Test in Infection Hypersensitivity, Proc. Soc. Exper. Biol. & Med. **80**: 69, 1952.
7. Tullis, J. L., and Rochow, E.: Non-wettable Surfaces, Blood **7**: 850, 1952.
8. Klein, E., Eridani, S., Djerassi, I., and Resnick, R.: A Simple Method for the Separation of Leukocytes From Whole Blood, Am. J. Clin. Path. (In press.)
9. Burnett, L.: Personal communication.
10. Tullis, J. L., editor: Blood Cells and Plasma Proteins, Their State in Nature, Cambridge, 1953, Harvard University Press, p. 272.
11. Wilson, T.: The Egestion of Phagocytosed Particles by Leukocytes, J. Exper. Med. **98**: 305, 1953.
12. Moeschlin, S., Meyer, H., Israels, L. G., and Tarr-Gloor, E.: Experimental Agranulocytosis. Its Production Through Leukocyte Agglutination by Antileukocytic Serum, Acta Haemat. **11**: 19, 1954.
13. Cecil, R., and Loeb, R.: Textbook of Medicine, ed. 8, Philadelphia, 1936, W. B. Saunders Company, p. 1032.
14. Boland, E. W., Headley, N. E., and Hurch, P. S.: Treatment of Agranulocytosis With Penicillin. Report of a Case Resulting From Chrysotherapy for Rheumatoid Arthritis, Proc. Staff Meeting, Mayo Clinic **21**: 197, 1946.
15. Bartch, E. C.: Agranulocytosis During Propylthiouracil Therapy, Am. J. Med. **5**: 48, 1948.
16. McManus, J. F.: Agranulocytosis Following Mapharsen Therapy, New England J. Med. **234**: 17, 1946.
17. Manganaro, F. J.: Malignant Granulocytopenia Treated With Penicillin, U. S. Naval Bull. **46**: 584, 1946.
18. Martensson, J., and Vikblach, I.: Idiopathic Immunoneutropenia. Report of a Case With a Leukocyte Agglutinating Factor in the Serum, Blood **9**: 632, 1954.

19. Hanson, K. B.: Immunologic Agranulocytosis, Acta med. scandinav. **145**: 169, 1953.
20. Moeschlin, S., Siegenthaler, W., Gasser, C., and Hässig, A.: Immunopancytopenia Associated With Incomplete Cold Hemagglutinins in a Case of Primary Atypical Pneumonia, Blood **9**: 214, 1954.
21. Kissmeyer-Nielson, F.: Demonstration of a Thrombocytopenic and a Leukopenic Factor in the Blood of Patients With Thrombocytopenia and Leukopenia, Acta Haemat. **9**: 337, 1953.
22. Miescher, P.: Leucopenie chronique par "auto-anticorps," Acta Haemat. **11**: 152, 1954.
23. Dubos, R.: Bacterial and Mycotic Infections of Man, ed. 2, Philadelphia, 1952, J. B. Lippincott Company, p. 106.

THE EXPERIMENTAL PRODUCTION OF HEINZ BODIES IN VARIOUS HEMOGLOBINOPATHIES AND THE THALASSEMIC SYNDROMES

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INTRAERYTHROCYTIC globules, commonly designated as Heinz bodies, were first described in an individual with potassium chlorate intoxication.[1] In 1890, Robert Heinz, studying the action of phenylhydrazine on the blood, produced similar structures, studied their behavior, and devised methods for their staining.[2-4] He characterized these bodies as irregularly shaped, refractile granules, which may remain stationary or demonstrate brownian motion. Occasionally they may be seen to extrude from the red cell or adhere to the membrane of the cell. The granules vary in size depending on the animal species studied and the drug used to produce them.

We undertook the present study to determine whether any difference in Heinz body formation could be related to variations in human hemoglobins, in view of their different globin moieties.

MATERIAL AND METHODS

The in vitro production of Heinz bodies was done with a modification of the method of Beutler and associates.[5] All patients studied had routine hematologic studies. Hemoglobin type was determined by paper and Tiselius electrophoresis[6] and alkali denaturation[7] for fetal (F) hemoglobin.

Three to 5 ml. of oxalated blood were obtained from each subject. The samples were used within 4 hours after venesection.

A buffer of pH 7.6 was prepared by mixing 8.7 parts 15 M Na_2HPO_4 and 1.3 parts 15 M KH_2PO_4.

Materials.—The drugs used were acetylphenylhydrazine, 100 mg. per cent; hydroxylamine hydrochloride, 40 mg. per cent; ascorbic acid, 50 mg. per cent; sulfanilamide, 200 mg. per cent; sodium hydrosulfite, 50 mg. per cent; sodium metabisulfite, 2 per cent solution. The buffer was used with all drugs except the last, when saline was employed.

Test Procedure.—One part of blood was left unaltered; a second portion was changed to reduce hemoglobin by exposure to CO_2 for 1 minute; the remainder had CO bubbled through until a cherry red color was obtained. To 2 ml. of the various drug solutions, 0.1 ml. of blood was added, and the mixture was incubated for 2 hours at 37° C. The tubes

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containing unaltered whole blood, i.e., oxygenated hemoglobin, were left uncapped. Those containing CO-hemoglobin were stoppered, and those containing reduced hemoglobin were incubated with CO_2 bubbling through the solutions. The procedure involving sodium metabisulfite differed in several respects. The solution was placed on a glass slide and one-third the amount of blood was added and mixed. This was allowed to remain at room temperature under glass for 10 minutes.

After incubation, 2 drops of crystal violet stain and 1 drop of the blood samples were mixed on a glass slide. After 5 minutes the specimens were examined microscopically.

All blood specimens underwent the same test procedure without exposure to the various chemical reagents or drugs. These made up the control samples.

Two hundred cells were counted and the percentage of cells containing Heinz bodies was recorded. The number of Heinz bodies within a single cell was estimated as follows: 1, 1+; 2 to 4, 2+; more than 4, 3+. All samples were counted in duplicate by 2 different observers.

RESULTS

Control Series.—Subjects with normal hemograms and whose cells contained normal adult (A) hemoglobin (10 patients; Table I).*

TABLE I. NORMAL ADULT (A) HEMOGLOBIN

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	98* (3+)†	96 (3+)	5‡ (1+)	Moderate size; stains deep-blue; reduced state, small and stains light-blue
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	95 (2 to 3+)	Moderate size; stains well
Sulfanilamide	70 (2+)	10 (1+)	70 (2+)	Small; stained well or were refractile
Ascorbic acid	95 (2 to 3+)	80 (2 to 3+)	2 (1+)	Dustlike
Sodium hydrosulfite	60 (1 to 2+)	7 (1+)	1 (1+)	Variable size and variable staining reaction
Sodium metabisulfite	80 (1+)	80 (1+)	Rare	Large, refractile, and often extracellular
Control	Negative	Negative	Negative	

*Figures denote percentage of cells containing Heinz bodies.
†Refers to number of Heinz bodies per positive cell.
‡In 3 patients, although all cells contained Heinz bodies, these were fewer in number per cell and had a different morphology.

Acetylphenylhydrazine was effective in producing Heinz bodies in 90 to 100 per cent of the cells whose hemoglobin was oxygenated or saturated with CO. Almost every cell had more than 4 granules (Fig. 1, *A*). These stained well and were moderate, about 2 μ in diameter, in size. Preparations containing reduced hemoglobin showed marked variations. Either only 5 per cent of the cells had Heinz bodies or, in 3 patients, although 90 per cent of the cells contained the granules, the average was 1+, and they were smaller and stained much more lightly.

*In all tables the first number represents percentage of cells containing Heinz bodies. The numbers in parentheses represent the number of Heinz bodies per cell.

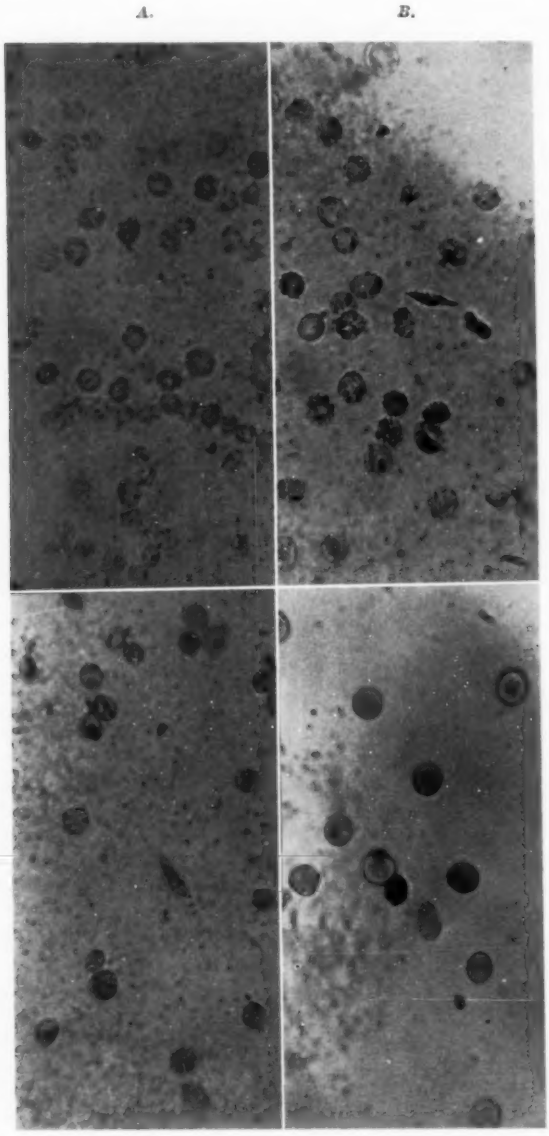

Fig. 1.—*A*, Normal red cells; oxygenated hemoglobin, acetylphenylhydrazine. *B*, Sickle-cell anemia; oxygenated hemoglobin, acetylphenylhydrazine. *C*, Sickle-cell anemia; oxygenated hemoglobin, hydroxylamine hydrochloride. *D*, Normal red cells; oxygenated hemoglobin, sodium hydrosulfite.

Hydroxylamine led to the formation of Heinz bodies in 90 to 100 per cent of the cells in all preparations. They were 3+ per cell, stained well, and were moderate in size.

Sulfanilamide gave a yield of Heinz bodies in 70 per cent of the cells whose hemoglobin was in the oxygenated or reduced state. When the preparations contained CO-hemoglobin, only 10 per cent of the cells showed small granules located at the cell periphery. These structures were always smaller than the comparable ones found with acetylphenylhydrazine. In a small percentage refractoriness to the stain was seen.

Heinz bodies were found in 80 to 95 per cent of the cells exposed to *ascorbic acid* with the hemoglobin in the oxygenated or CO state. These were dustlike in appearance and averaged 2+ per cell; with reduced hemoglobin less than 2 per cent of the cells were positive.

Heinz bodies produced by *sodium hydrosulfite* were extremely variable in size and staining properties (Fig. 1, *D*). When the hemoglobin was oxygenated, 60 per cent of the cells contained the granules, whereas the preparations with reduced hemoglobin or CO-hemoglobin yielded less than 10 per cent positive cells.

Sodium metabisulfite gave 80 per cent positive results with oxygenated and CO-hemoglobin, but only rarely were intraerythrocytic granules found in cells under constant CO_2 exposure. These Heinz bodies were almost uniformly refractile and were often seen adhering to the cell membrane or in the form of stalklike extrusions.

Control samples under the same test conditions, without exposure to the various chemical agents, gave uniformly negative results.

TABLE II. S-HEMOGLOBIN: SICKLE-CELL ANEMIA SERIES

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	100 (3+)	100 (3+)	5 (1+)	Moderate size; stains deep-blue; reduced state, small and stains light-blue
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	100 (3+)	Moderate size; stains well
Sulfanilamide	50 (2+)	50 (2+)	4 (1+)	Small; stains well, a few remaining refractile
Ascorbic acid	95 (3+)	85 (2+)	50 (2+)	Dustlike
Sodium hydrosulfite	6 (1+)	7 (1+)	4 (1+)	Variable size and variable staining reactions
Sodium metabisulfite	Very rare	Negative	Negative	Refractile
Control	Negative	Negative	Negative	

Sickle-Cell Anemia Series.—This series consisted of subjects with 80 to 95 per cent S-hemoglobin and 5 to 20 per cent fetal (F) hemoglobin (5 patients; Table II). When *acetylphenylhydrazine* was employed, 100 per cent of the cells

whose hemoglobin was oxygenated or in the CO form contained Heinz bodies (Fig. 1, *B*). Reduced hemoglobin preparations gave 3 to 10 per cent positive yields. These bodies were comparable to those found with normal hemoglobin.

Hydroxylamine again produced Heinz bodies in 100 per cent of the cells under all conditions. No difference was noted between these and the ones seen with normal red cells (Fig. 1, *C*).

Sulfanilamide produced Heinz bodies in 50 per cent of the cells with oxygenated or CO-hemoglobin. CO_2 exposure reduced the yield to less than 5 per cent. These bodies were uniformly small and occasionally did not stain well, remaining refractile.

Ascorbic acid led to the formation of granules in 80 to 100 per cent of the cells with oxygenated or CO-hemoglobin, but only in 50 per cent of the cells incubated with CO_2. They were again in the form of blue specks.

Sodium hydrosulfite never produced granules in more than 10 per cent of the cells regardless of the hemoglobin state. These were variable in size and staining properties.

Sodium metabisulfite was essentially without effect; it led to the formation of a refractile body in a few red cells.

Controls were again negative.

Fetal Hemoglobin Series.—Cord blood was obtained from newborn infants. The average fetal (F) hemoglobin content was 70 per cent, the remainder being A-hemoglobin (5 subjects; Table III).

TABLE III. F-HEMOGLOBIN: CORD BLOOD SPECIMENS

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	100 (3+)	100 (3+)	50 (1 to 2+)	Moderate to large size; stains well
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	100 (3+)	Moderate size; stains well
Sulfanilamide	15 (1 to 2+)	10 (1+)	15 (1+)	Small size; stains deep-blue or remains refractile
Ascorbic acid	100 (3+)	100 (3+)	100 (3+)	Dustlike
Sodium hydrosulfite	10 (1+)	8 (1+)	7 (1+)	Variable size and staining properties
Sodium metabisulfite	18 (1+)	15 (1+)	Rare	Refractile; often stalklike extrusions
Control	Negative	Negative	Negative	

Hydroxylamine was effective in all preparations, all cells containing intraerythrocytic granules. No difference in morphologic characteristics was noted when compared to similar blood samples containing only hemoglobin-A.

Intraerythrocytic bodies were found in 10 to 15 per cent of the red cells exposed to *sulfanilamide*. The state of the hemoglobin did not affect the staining properties of the globules which were small and either stained deep-blue or remained refractile.

Ascorbic acid was effective in the formation of blue, dustlike granules in each cell of the various blood samples.

From 5 to 15 per cent of the cells incubated with *sodium hydrosulfite* contained granules which were variable in size and staining properties. Altering the hemoglobin state did not significantly affect the results.

Sodium metabisulfite was without effect in cells with reduced hemoglobin, but produced Heinz bodies in 15 to 18 per cent of the other preparations. The granules remained refractile.

Control samples were negative.

TABLE IV. A-THALASSEMIA

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	100 (3+)	100 (3+)	–	Moderate size; stains well
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	–	Moderate size; stains well
Sulfanilamide	50 (2+)	60 (1+)	–	Small size; stains deep-blue
Ascorbic acid	100 (2+)	70 (1 to 2+)	–	Variable size, but predominantly small; stains light-blue
Sodium hydrosulfite	20 (1+)	30 (1+)	–	Variable size; about half the cells remain refractile
Sodium metabisulfite	80 (1+)	80 (1+)	–	Refractile; large
Control	10 (1+)	20 (1+)	–	Moderate size; stains well

TABLE V. S-THALASSEMIA

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	100 (3+)	100 (3+)	100 (2+)	Small to moderate size; stains well
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	100 (3+)	Small size; stains well
Sulfanilamide	80 (2+)	80 (1+)	50 (1+)	Small size, stains well; reduced state, few remain refractile
Ascorbic acid	100 (3+)	100 (3+)	70 (1 to 2+)	Dustlike
Sodium hydrosulfite	90 (2+)	100 (2+)	80 (1+)	Small size; few remain refractile
Sodium metabisulfite	70 (2+)	–	50 (2+)	Refractile and often with stalks
Control	30 (1+)	40 (1+)	8 (1+)	Small to moderate size; stains well

Thalassemia Series.—One patient each with S-thalassemia, C-thalassemia, and A-thalassemia was studied. The latter, a patient with homozygous Mediterranean anemia, had been splenectomized one year previously in a successful

attempt to ameliorate an extracorpuscular hemolytic process. The patient had not had any transfusion for 4½ months prior to the time of the test procedure. (Tables IV, V, and VI.)

TABLE VI. C-THALASSEMIA

CHEMICAL	GASEOUS STATE			MORPHOLOGY
	O_2	CO	CO_2	
Acetylphenylhydrazine	100 (3+)	100 (3+)	100 (3+)	Small to moderate size; stains well
Hydroxylamine hydrochloride	100 (3+)	100 (3+)	100 (3+)	Small size; stains well
Sulfanilamide	33 (1+)	15 (1+)	10 (1+)	Small size; stains faintly blue
Ascorbic acid	100 (3+)	75 (3+)	90 (3+)	Very small; light-blue
Sodium hydrosulfite	15 (1+)	10 (1+)	15 (1+)	Small size; stains faintly blue
Sodium metabisulfite	–	–	–	
Control	25 (1+)	25 (1+)	20 (1+)	Small to moderate size; a few remain refractile

The outstanding difference in these 3 patients was the appearance of Heinz bodies in each of the control preparations. These intraerythrocytic granules were moderate in size, stained well, and each positive cell rarely contained more than 1 or 2 granules. No explanation was found for the diminished yield of Heinz bodies when sulfanilamide or sodium hydrosulfite was used as the test chemical in the patient with hemoglobin C-thalassemia.

DISCUSSION

The numerous theories concerning the origin, composition, and significance of Heinz bodies have been amply reviewed by Webster[8] and Fertman and Fertman.[9] More recently, Beutler and associates[10] have put forth the concept that Heinz body production is inversely related to the glutathione content of the red cells in primaquine-sensitive individuals. Inhibition of sulfhydryl compounds (glutathione) caused nonsensitive red cells to behave like primaquine-sensitive cells with increased production of Heinz bodies.

There is no unanimity concerning the nature of Heinz bodies. We undertook the present study to determine whether the formation of these granules in red cells with different hemoglobins might throw some light upon this question. In their study of primaquine sensitivity, Beutler and co-workers[11] could not detect any differences in patients with normal hemoglobin, sickle-cell trait, sickle-cell anemia, hemoglobin-C disease, or thalassemia.

Acetylphenylhydrazine, an oxidizing reagent, was effective in producing Heinz bodies in 90 to 100 per cent of the red cells in every blood sample tested when the hemoglobin was oxygenated or in the CO form. We were unable to confirm the observations of Beutler and associates[5] regarding the number of

Heinz bodies in red cells exposed to this drug. They found that cells from patients who were primaquine sensitive had 5 or more of the intraerythrocytic granules per cell, whereas those from normal subjects rarely had more than 1 or 2. In all of our acetylphenylhydrazine preparations the Heinz bodies averaged more than 4 per cell. Introduction of CO_2 into the test system invariably decreased the yield except in the patient with C-thalassemia disease.

That oxygen availability is not a crucial factor in the production of Heinz bodies seems clear from the experiments utilizing hydroxylamine hydrochloride. This drug, a reducing agent, was equally effective in their production regardless of the state of the different hemoglobins. All other drugs employed reacted variably to the introduction of CO or CO_2 into the test system.

The morphologic characteristics of the Heinz bodies produced by each reagent remained fairly constant in the different blood samples. An exception to this was the appearance of smaller or more lightly stained intraerythrocytic granules when red cells were exposed to acetylphenylhydrazine under continuous CO_2 flow. Variability in the staining property was observed mainly in the Heinz bodies produced by sulfanilamide and sodium hydrosulfite, some staining well and others remaining refractile.

The observation has been made by Fisher,[12] and confirmed by us, that sodium metabisulfite has little effect in cells with S-hemoglobin but produces Heinz bodies in normal cells. He postulated that differences in globin moieties accounted for this finding. The ability of hydroxylamine hydrochloride, another reducing agent, to produce Heinz bodies in 100 per cent of red blood cells from patients with sickle-cell anemia, even under constant CO_2 flow, would seem to contradict this hypothesis.

In 1954, Minnich and associates[13] found Heinz bodies in 4 of 32 patients with Mediterranean anemia. Three of our patients, one each with S-thalassemia, C-thalassemia, and A-thalassemia, had Heinz bodies in their red cells without exposure to any chemical. Our study is too small to correlate the appearance of Heinz bodies to the hemolytic process, although the patient with the most severe hemolysis (A-thalassemia) had the fewest number of cells with these granules. It is interesting to note that this patient had undergone a splenectomy a year previously and splenectomy seems to enhance the appearance of Heinz bodies in the peripheral circulation in susceptible individuals.[14]

SUMMARY

The production of Heinz bodies utilizing several chemical reagents in cells containing predominantly A-, S-, and F-hemoglobins was studied. The effect of oxygen, carbon monoxide, and carbon dioxide was also noted.

The morphology of these granules was characteristic for each chemical employed.

Three patients with thalassemic syndromes showed spontaneous Heinz body formation.

Erythrocytes in the shape of sickle cells can form Heinz bodies.

The ability to form Heinz bodies does not appear to be a function of any particular hemoglobin type tested. The presence of the gene for thalassemia may predispose the individual to the formation of these granules.

REFERENCES

1. Riess, L.: Über Vergiftung mit chlorsaurem Kalium, Berl. klin. Wchnschr. **19**: 785, 1882.
2. Heinz, R.: Die praktische Verwendbarkeit von Phenylhydrazinderivaten als Fiebermittel, Berl. klin. Wchnschr. **27**: 47, 1890.
3. Heinz, R.: Morphologische Veränderungen der roten Blutkörperchen durch Gifte, Virchows Arch. f. path. Anat. **122**: 112, 1890.
4. Heinz, R.: Handbuch der Experimentellen Pathologie und Pharmakologie, Jena, 1904, G. Fischer, vol. 1, pp. 336 and 396.
5. Beutler, E., Dern, R. J., and Alving, A. S.: The Hemolytic Effect of Primaquine. VI. An In Vitro Test for Sensitivity of Erythrocytes to Primaquine, J. Lab. & Clin. Med. **45**: 40, 1955.
6. Singer, K.: Hereditary Hemolytic Disorders Associated With Abnormal Hemoglobins, Am. J. Med. **18**: 633, 1955.
7. Singer, K., Chernoff, A. I., and Singer, L.: Studies on Abnormal Hemoglobins. I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation, Blood **6**: 413, 1951.
8. Webster, S. H.: Heinz Body Phenomenon in Erythrocytes, Blood **4**: 479, 1949.
9. Fertman, M. H., and Fertman, M. A.: Toxic Anemias and Heinz bodies, Medicine **34**: 131, 1955.
10. Beutler, E., Dern, R. J., Flanagan, C. L., and Alving, A. S.: The Hemolytic Effect of Primaquine. VII. Biochemical Studies of Drug-Sensitive Erythrocytes, J. Lab. & Clin. Med. **45**: 286, 1955.
11. Beutler, E., Dern, R. J., and Alving, A. S.: The Hemolytic Effect of Primaquine. III. A Study of Primaquine-Sensitive Erythrocytes, J. Lab. & Clin. Med. **44**: 177, 1954.
12. Fisher, B.: The Production of Heinz Bodies in Normal Human Erythrocytes by Metabisulfite, Science **118**: 631, 1953.
13. Minnich, V., Na-Nakorn, S., Chongchareonsuk, S., and Kochaseni, S.: Mediterranean Anemia. A Study of Thirty-two Cases in Thailand, Blood **9**: 1, 1954.
14. Selwyn, J. G.: Heinz Bodies in Red Cells After Splenectomy and After Phenacetin Administration, Brit. J. Haemat. **1**: 173, 1955.

SERUM ALDOLASE IN THE NEONATAL PERIOD: INCLUDING A COLORIMETRIC DETERMINATION OF ALDOLASE BY STANDARDIZATION WITH DIHYDROXYACETONE

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IN RECENT years much attention has been given to the study of serum and plasma enzymes in disease, and, among these, serum aldolase. Aldolase is the enzyme which takes part in the intermediary breakdown of glucose at the level of fructose-1,6-diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Elevation in serum aldolase levels was reported in hepatitis,[1, 2] prostatic cancer,[3, 4] myopathies,[5, 6] and in acute myocardial infarction.[7]

Notwithstanding the increasing interest in aldolase activity in the past few years, there has been no data reported on enzyme levels in the neonatal period. The present study included serum and plasma aldolase levels of mothers at delivery, the cord blood, and during the first 4 days of the newborn infant.

Serum aldolase was determined by a modification of the method of Bruns[8] so as to be used as a macromethod or a micromethod where the amounts of serum or plasma were reduced to 1:10 quantities, making the method applicable for newborn infants and small animals. Beck[9] has shown experimentally that the color development for both trioses, glyceraldehyde and dihydroxyacetone, is similar in a 60-minute period and with such a time-color development either triose may be used as a secondary standard in place of the more complicated alkali-labile P used heretofore. In the present method we have used dihydroxyacetone as such a secondary standard and all readings are recorded in "dihydroxyacetone units."

During the preparation of the reference curves with dihydroxyacetone it was further observed that the color developed was not linear in the higher ranges such as those found in hepatitis and in the normal range of the newborn infant.

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MATERIALS AND METHODS

Reagents.—

Fructose-1,6-diphosphate (0.05 M, pH 7.4): The commercial barium salt* was used without further purification. Dissolve 0.4 Gm. of the salt in 8 ml. of 1 N HCl and precipitate the barium by dropwise addition of saturated Na_2SO_4. The precipitation is carried out in a centrifuge tube with intermittent centrifugation. The reaction is complete when no further precipitate forms (about 0.4 ml. of the sulfate is required). The $BaSO_4$ is removed by centrifugation and the supernatant adjusted to pH 7.4 by the addition first of NaOH pellets and then dropwise 1 N NaOH. Make to 10 ml. with water. When prepared in this way the molarity of the substrate was found to be close to 0.05 M for fructose as determined by the method of Roe.[10]

Hydrazine (0.56 M, pH 7.4): Dissolve 7.28 Gm. hydrazine sulfate in 50 ml. water and adjust to pH 7.4 with NaOH. Make to 100 ml. with water.

γ-Collidine buffer (0.1 M, pH 7.4): Dissolve 1.21 Gm. γ-collidine in 50 ml. water, adjust to pH 7.4 with HCl, and make to 100 ml. with water.

Iodoacetic acid (0.002 M, pH 7.4): Dissolve 40 mg. CH_2ICOOH in 50 ml. water, adjust to pH 7.4 with dilute NaOH, and make to 100 ml. with water.

2,4-Dinitrophenylhydrazine: One-tenth gram dissolved in 100 ml. of 2 N HCl.

Sodium hydroxide: 0.75 N.

Trichloroacetic acid: 10 per cent.

Macroprocedure.—The reactants are added to a test tube in the following order: 1.0 ml. collidine buffer, 1.0 ml. serum or plasma, 0.25 ml. hydrazine, 0.25 ml. iodoacetate, and 0.25 ml. fructose-1,6-diphosphate substrate. Incubate sample for 60 minutes at 38° C. Add 5.0 ml. of 10 per cent trichloroacetic acid, shake well, and filter or centrifuge. A blank is prepared simultaneously by omitting the fructose substrate but adding it after the trichloroacetic acid. No incubation for the blank is necessary.

The filtrates from the sample and blank are then treated as follows: to 1.0 ml. filtrate add 1.0 ml. of 0.75 N NaOH and let stand at room temperature for 10 minutes. Then add 1.0 ml. of 2,4-dinitrophenylhydrazine and let stand 60 minutes at 38° C. Finally, add 7.0 ml. of 0.75 N NaOH, mix well, and read in 8 minutes at 540 mμ with the blank set at 100 per cent transmittance. The "dihydroxyacetone units" are obtained from a reference curve.

TABLE I. COMPARISON OF MACRO- AND MICROPROCEDURE FOR SERUM ALDOLASE. VALUES RECORDED IN "DIHYDROXYACETONE UNITS"

MACROPROCEDURE	MICROPROCEDURE	PER CENT DIFFERENCE
18.8	18.6	−1.1
22.4	20.3	−9.4
23.3	23.3	0
27.2	27.3	+0.4
30.6	30.4	−0.7
39.4	38.7	−1.8
40.9	43.4	+6.1
54.1	53.9	−0.4

Microprocedure.—This procedure is carried out as above except that quantities of reactants for incubation are reduced to 1:10 and the reaction carried out in micro-test tubes. Whereas in the macromethod 1.0 ml. filtrate is made to a final dilution of 10 ml.,

*Schwarz Laboratories, Inc., Mt. Vernon, N. Y.

in this case 0.4 ml. filtrate is made to a final dilution of 4 ml., giving the same concentration of color for both procedures and permitting the use of the same reference curve (Table I).

Preparation of a Reference Curve.—A stock solution of dihydroxyacetone is prepared by dissolving 200 mg. in 100 ml. water.* The stock solution of 2 mg. per milliliter is kept in the refrigerator for 48 to 72 hours to complete depolymerization[9] after which further dilutions are made so that 1 ml. contains from 2 mg. down to 0.1 mg. dihydroxyacetone. In preparing the standard curve it is essential that the method be followed as described in the macroprocedure except that 1 ml. of dihydroxyacetone solution is used in place of the serum, other reagents and steps being identical. A plot of absorbance against milligrams of dihydroxyacetone is shown in Fig. 1. These results were obtained by the use of a Beckman DU spectrophotometer at 540 mμ with 1.0 cm. cuvettes. The lower scale of the abscissa gives the units and we have defined the "dihydroxyacetone unit" as the amount of color developed by 0.01 mg. dihydroxyacetone under the conditions of this procedure.

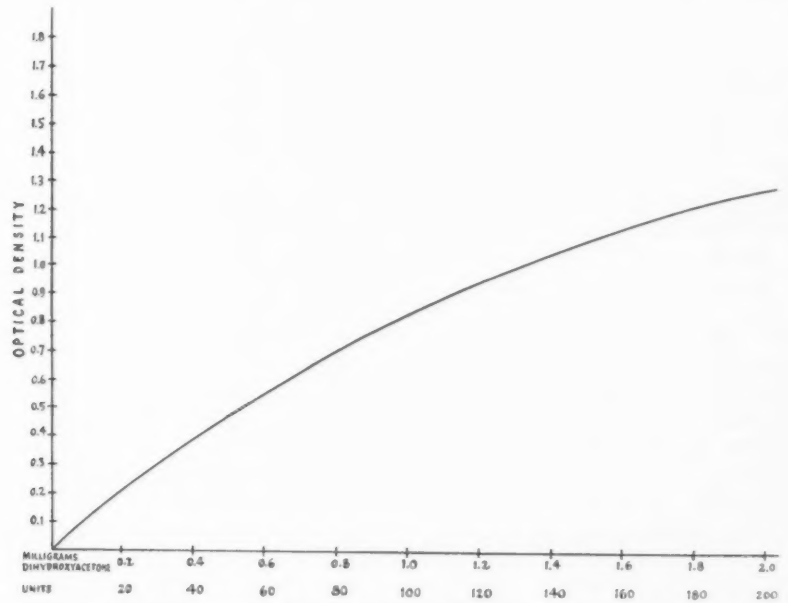

Fig. 1.—Calibration curve for aldolase activity in "dihydroxyacetone units." (See text.)

Replicate determinations usually checked to within ±3 per cent. A study of color fading showed that the absorbance decreased at the rate of 1 per cent for every 3 minutes of standing.

The time of enzyme assay was also investigated. Sibley and Lehninger[11] selected 30 minutes as the period of incubation. The assay in this procedure was extended to 60 minutes to intensify the color and permit the use of smaller samples. That 60 minutes is still in a linear range is shown in Table II. The temperature of incubation should be thermostatically controlled.

*We used dihydroxyacetone samples obtained from the Mann Research Laboratories and the Krishell Laboratories with similar results.

TABLE II. SERUM ALDOLASE ACTIVITY AS A FUNCTION OF TIME OF INCUBATION. VALUES RECORDED IN "DIHYDROXYACETONE UNITS"

INCUBATION		60 MINUTES
30 MINUTES	60 MINUTES	30 MINUTES
3.8	8.1	2.13
6.7	13.3	1.99
8.6	16.8	1.95
12.2	23.4	1.92
15.5	32.8	2.12

RESULTS

The study for normal levels included 75 adults, divided into 27 men and 48 women. Blood was taken by venipuncture, permitted to clot, and centrifuged; sera that were visibly hemolyzed were discarded. Table III gives a résumé of these normal values. The average for men was found significantly higher than women, with $t = 2.12$ and P less than 0.05.[12]

TABLE III. SUMMARY OF SERUM ALDOLASE IN ADULTS. VALUES RECORDED IN "DIHYDROXYACETONE UNITS"

DETERMINATIONS	MEN (27)	WOMEN (48)
Total range	7.2-21.3	6.1-19.2
Average	14.3	12.3
S.D.	± 4.3	± 3.7

The data herein presented consist of two separate studies in the neonatal period; first, the mother and cord blood drawn simultaneously at delivery, and second, the blood levels during the first 4 days of newborn life. Blood was withdrawn from the mother by venipuncture immediately after delivery and from the cord without any attempt to distinguish venous from arterial blood. After clot retraction the specimen was centrifuged and the serum separated. The sample was discarded when any visible hemolysis was apparent. Blood specimens were obtained from the newborn infants at varying times from a few hours after birth through the fourth day. The heel was punctured by a Bard-Parker blade and blood drawn into a capillary tube coated with heparin. The tubes were centrifuged and the plasma column separated into small test tubes. The aldolase was determined on 0.1 or 0.05 ml. plasma by the microprocedure described above. The results are recorded in "dihydroxyacetone units" obtained from a reference curve.

TABLE IV. SUMMARY OF SERUM ALDOLASE OF CORD AND MATERNAL BLOOD COMPARED WITH ALDOLASE LEVELS IN WOMEN. VALUES RECORDED IN "DIHYDROXYACETONE UNITS"

	WOMEN*	CORD	MOTHER	CORD / MOTHER
Subjects	48	46	46	46
Total range	6.1-19.0	12.6-84	7.1-43.8	0.79-5.00
Average	12.3	40.1	19.6	2.7
S.D.	± 3.7	±18.1	± 7.8	

*Nonpregnant.

Table IV gives a résumé of values obtained in mother and cord blood of 46 subjects. Most patients delivered spontaneously and no significant difference was found in those delivered by forceps or cesarean section. The

average for the serum from cord blood was 40.1 ± 18.1 units while for the mother's blood it was found to be 19.6 ± 7.8 units. The average value of 19.6 units for mother's serum at delivery compares with 12.3 for women in the child-bearing ages. The mothers' values are significantly higher than nonpregnant women with t = 5.80 and P less than 0.01. The distribution of the serum levels in the 3 groups, nonpregnant, mothers at delivery, and the cord blood, are shown in Fig. 2.

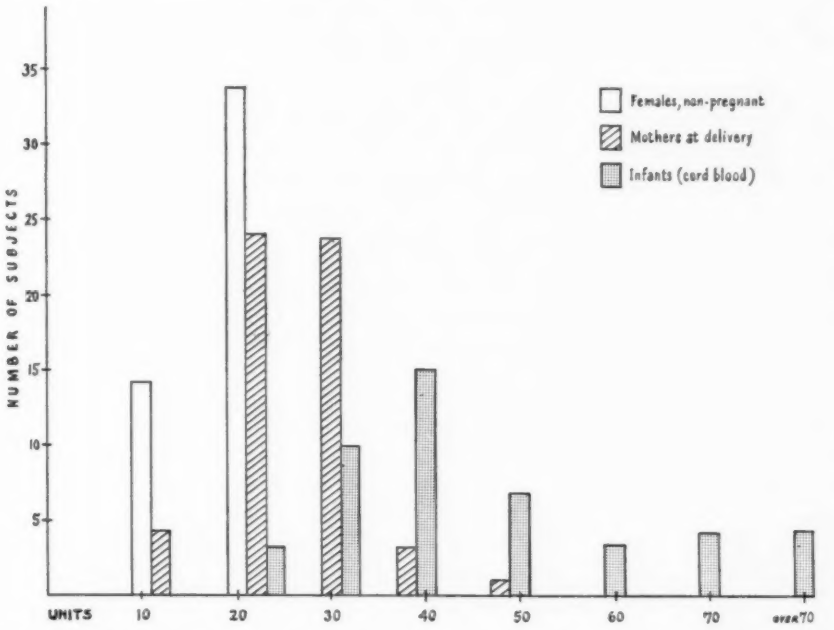

Fig. 2.—Distribution of serum aldolase in control, mother, and cord blood. Values recorded in "dihydroxyacetone units."

The enzyme levels in the newborn were particularly interesting in that they were about twice as high as the cord blood and about 5 times the level in adults. The data for this group may be seen in Table V. In this series of experiments, 104 analyses were carried out on 89 newborn infants. Some specimens were obtained as soon as 4 hours after birth (recorded as the first day) and the others up to the fourth day. A comparison of the daily levels shows no significant difference from the first to the fourth day.

Since these specimens were obtained by heel puncture the possibility of tissue destruction and enzyme elaboration in situ was considered. For that reason a series of adults was used as controls where blood was obtained by finger puncture using the same manipulation as in the heel puncture of the newborn infant. The average enzyme activity of heparinized plasma obtained

by finger puncture from 28 adults, men and women combined, was 15.5 ± 5.7 units, only slightly higher than the levels in adult sera by venipuncture. These finger puncture data are included in Table V.

TABLE V. SUMMARY OF PLASMA ALDOLASE IN THE FIRST FOUR DAYS OF THE NEONATAL PERIOD COMPARED WITH ALDOLASE LEVELS IN ADULTS. VALUES RECORDED IN "DIHYDROXYACETONE UNITS"

	ADULTS*	NEWBORN INFANTS (DAYS AFTER BIRTH)			
		1	2	3	4
Subjects	28	25	30	22	27
Total range	7.2-25.4	49.3-111.5	47.1-116.5	49.6-119.5	42.9-120.0
Average	15.5	75.6	77.6	78.7	70.7
S.D.	± 5.7	±18.4	±19.6	±21.4	±20.5

*Plasma obtained by finger puncture from heparinized blood.

DISCUSSION

The data of this study suggest various questions which can be dealt with only by theorizing. The level of serum aldolase found in women during labor is significantly higher than that in nonpregnant women in the childbearing age group. The present concept is that serum aldolase increases in diseases associated with tissue destruction or necrosis, i.e., hepatitis, myocardial infarction, and muscular dystrophy. No significant tissue destruction nor necrosis can be assumed to occur at the time of labor and delivery. However, one must consider the occasional use of obstetrical forceps and the frequency of episiotomy and repair. Since aldolase is a glycolytic enzyme, the increased muscular activity during labor may be the source of the increased serum aldolase level.

The difference between the mother's blood and the cord blood, as seen in Table IV, raises the obvious question of placental permeability. The cord blood aldolase is at a significantly higher level, so that the mother and fetus are not in enzyme equilibrium. A parallel finding has been reported by Glendening and associates[13] in their study of transaminase in pregnant women, where approximately twice as much of this enzyme was present in cord blood as compared to maternal blood. The placenta at term may act as a selective membrane, a storage site, or a primary source of these enzymes. Most of the known enzymes have been identified in the placenta.

Another observation of interest is the much higher serum aldolase activity in the newborn infant as compared to the level in the normal adult, and, more directly, when compared to the cord blood at time of birth. Determinations in the newborn infant carried out as early as 4 hours after birth already showed these much higher levels (as compared to the cord blood). Here, in newborn life, without any obvious concomitant process of tissue destruction or necrosis, the enzyme activity is as high as that seen in hepatitis, myocardial infarction, muscular dystrophy, or in tumor-bearing animals.

One of the concepts proposed for the increased serum aldolase in pathologic states is that the enzyme is liberated from areas of cellular destruction or necrosis.[7] Hepatitis, myocardial infarction, and myopathies are associated

with cellular breakdown. However, in the newborn infant, the same elevated enzyme levels are obtained in a physiologic state, for there is no known sudden tissue breakdown which occurs at birth and which can be compared with the clinical states above mentioned. One may, therefore, postulate that the homeostasis for glycolytic enzymes in the newborn infant requires these higher levels of aldolase as part of the normal response to extrauterine existence.

The observation, in this study, of a relatively high aldolase level in newborn life is particularly interesting. Our experience with tributyrinase,[14] cholinesterase,[15] and arginase[15] has indicated no significant difference between newborn life and the adult. An elevation of transaminase in the cord blood has already been noted.[13]

One may assume in the case of aldolase that there is some underlying glycolytic process in the newborn infant which requires a higher enzyme level, possibly for increased muscular activity. It is yet to be determined when the newborn infant's level of aldolase returns to known adult levels, or which, if any, pathologic newborn states show an increase or decrease.

The activity of the adrenal glands in newborn life may play a role in the resulting increased level of aldolase.[16] The adrenal glands at birth are 20 times the relative normal adult size. Schapira[17] has demonstrated in rabbits increased plasma aldolase after cortisone and ACTH administration. Weber and associates[18] have described increased liver glucose-6-phosphatase activity after cortisone administration. It is possible, therefore, that the higher level of aldolase in the newborn infant may be regulated by the adrenal cortical hormones as part of the specific physiologic requirements of glycogen and sugar metabolism present at time of birth and in the neonatal period.

SUMMARY

A method is described for the estimation of serum aldolase activity based on the hydrolysis of fructose-1,6-diphosphate and the color development of the resulting trioses. A secondary standard of dihydroxyacetone is used for establishing a reference curve and units of activity. The method is applicable for amounts of serum as low as 0.1 ml., or less where enzyme activity is increased.

The range for normal adult values with this method is presented, and the levels for blood serum of men are shown to be significantly higher than for women.

A study of plasma and serum aldolase in the neonatal period was carried out on mother's blood, cord blood, and in the first 4 days of newborn life.

The enzyme levels of cord blood were significantly higher than those of mothers' taken at delivery, while the activity of plasma aldolase was highest in the newborn infant.

The possible significance of these results is discussed.

REFERENCES

1. Sibley, J. A.: The Clinical Significance of Serum Aldolase, Proc. Staff Meet., Mayo Clin. **29**: 591, 1954.

2. Bruns, F., and Jacob, W.: Studien über Serumenzyme bei Erkrankungen der Leber. Die Aktivität der phosphohexoseisomerase, Aldolase, Klin. Wchnschr. **32**: 1041, 1954.
3. Baker, R., and Govan, D.: The Effect of Hormonal Therapy of Prostatic Cancer on Serum Aldolase, Cancer Res. **13**: 141, 1953.
4. Baker, R., Govan, D., Huffer, J., and Cason, J.: Biological Titration of Diethylstilbestrol Against Activity of Prostatic Cancer: Effect on Serum Aldolase, J. Clin. Endocrinol. **13**: 383, 1953.
5. Schapira, G., Dreyfus, J. C., and Schapira, F.: L'élevation du taux de l'aldolase sérique; test biochimique des myopathies, Semaine hôp. Paris **29**: 1917, 1953.
6. Dreyfus, J. C., Schapira, G., and Schapira, F.: Biochemical Study of Muscle in Progressive Muscular Dystrophy, J. Clin. Invest. **33**: 794, 1954.
7. Volk, B. W., Losner, S., Aronson, S. M., and Lew, H.: The Serum Aldolase Level in Acute Myocardial Infarction, Am. J. M. Sc. **232**: 38, 1956.
8. Bruns, F.: Bestimmung und Eigenschaften der Serumaldolase, Biochem. Ztschr. **325**: 156, 1954.
9. Beck, W. S.: Determination of Triose Phosphates and Proposed Modifications in the Aldolase Method of Sibley and Lehninger, J. Biol. Chem. **212**: 847, 1955.
10. Roe, J. H.: A Colorimetric Method for the Determination of Fructose in Blood and Urine, J. Biol. Chem. **107**: 15, 1934.
11. Sibley, J. A., and Lehninger, A. L.: Determination of Aldolase in Animal Tissues, J. Biol. Chem. **177**: 859, 1949.
12. Mainland, D.: Elementary Medical Statistics, Philadelphia, 1952, W. B. Saunders Company.
13. Glendening, M. B., Cohen, A. M., and Page, E. W.: Influence of Pyridoxine on Transaminase Activity of Human Placenta, Maternal and Fetal Blood, Proc. Soc. Exper. Biol. & Med. **90**: 25, 1955.
14. Friedman, M. M., and Shriro, R. L.: Plasma Tributyrinase in the Neonatal Period, Clin. Chem. **2**: 407, 1956.
15. Unpublished data.
16. Brenner, M. D.: Studies on the Involution of the Fetal Cortex of the Adrenal Gland, Am. J. Path. **16**: 787, 1940.
17. Schapira, F.: Hyperaldolasémie plasmatique provoquée par la cortisone et l'ACTH, Compt. rend. Soc. biol. **148**: 1997, 1954.
18. Weber, G., Allard, C., DeLamirande, G., and Cantero, A.: Increased Liver Glucose-6-phosphatase Activity After Cortisone, Biochem. et biophys. acta **16**: 618, 1955.

THE SURVIVAL OF Cr^{51}-LABELED AUTOGENOUS ERYTHROCYTES IN CHILDREN

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THE advantages, disadvantages, and interpretation of Cr^{51}-labeled erythrocyte survival time in adults have been adequately documented.[1-6] However, we were not able to find any published reports of Cr^{51}-labeled erythrocyte survival time in children. Previous studies of survival time, employing the Ashby technique, in normal children have utilized adult donor cells.[7-12] We did not feel justified in assuming that values obtained by using donor cells from children would necessarily be the same as in adults. Hence, the present studies were performed.

MATERIALS AND METHODS

A group of clinically healthy children was not available for study. Therefore, our subjects consisted of children convalescing from a variety of acute infectious diseases, who were demonstrated to have a normal hematocrit, normal blood smear, and normal reticulocyte count (in 3 children it was slightly elevated at the time the study was initiated).

Approximately 10 ml. of blood was drawn from each subject and placed in a sterile siliconed glass vial* containing acid citrate dextrose solution and Cr^{51}* (1.0 μc/Kg.). The mixture was allowed to incubate for 1 hour at room temperature and was then reinjected into the subject. An initial blood sample was obtained 1 hour after injection and subsequent samples were obtained 24 hours later and at weekly intervals thereafter.

Two milliliter samples of blood were pipetted quantitatively, in duplicate, into Lusteroid tubes for assay of radioactivity. Prior to assay the blood samples were hemolyzed by freezing and thawing. Two milliliter samples of plasma from the initial and 24-hour samples were assayed for radioctivity; subsequent samples of plasma were not counted, because plasma samples have previously been found to be free of significant radioactivity by this time.[13] Radioactivity was assayed in a well-type scintillation counter for a period of time sufficient to ensure a counting error of less than 1.5 per cent. Microhematocrits were done in duplicate. All samples were corrected to the hematocrit of the initial sample, but not for plasma trapping. The initial postinjection and 24-hour samples were corrected for plasma radioactivity. Survival time was determined by assaying samples simultaneously with the initial postinjection sample and expressing residual radioactivity as a percentage of the initial sample.

The data were plotted on semilogarithmic paper with percentage initial radioactivity as the ordinate (logarithmic scale) and the time in days as the abscissa (linear scale).

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*Obtained from Abbott Laboratories, North Chicago, Ill.

A line was drawn, from the initial postinjection specimen as the origin, by visual inspection through the data and the point at which the blood radioactivity decreased to 50 per cent of the initial radioactivity determined.

RESULTS

The study was initiated in 32 patients and followed at least until radioactivity fell to less than half of the initial specimen level in 15. The 24-hour percentage of initial radioactivity, time of half initial radioactivity, and pertinent clinical data are tabulated in Table I. The average 24-hour radioactivity was 96.5 per cent. Thirteen patients had half initial radioactivity times in the range of 25 to 40 days. The remaining 2 had half times of 24 days.

TABLE I. CLINICAL AND EXPERIMENTAL DATA

PATIENT	AGE	SEX	CONVALESCENT FROM	HEMATOCRIT	RETICULOCYTE COUNT (PER CENT)	24-HOUR PER CENT INITIAL RADIOACTIVITY	TIME OF HALF INITIAL RADIOACTIVITY (DAYS)
F. S.	2½	F	TBC Meningitis	40	2.2	97.1	35
C. P.	3½	F	Scarlet fever	36	2.0	100.7	31
L. P.	5	F	Scarlet fever	36	1.3	95.6	28
Re. R.	5	F	Scarlet fever	36	0.8	98.1	31
Ra. R.	5	M	Mumps	34	1.1	96.5	24
S. F.	6	M	Scarlet fever	37	2.0	88.6	31
R. J.	7	F	TBC Meningitis	36	1.0	101.5	29
J. H.	7	M	Varicella	36	--	95.7	40
W. J.	7	M	Scarlet fever	35	1.0	100.0	33
L. D.	7	F	H. influenza Meningitis	36	1.1	95.1	24
P. J.	7	F	Rubeola	37	0.5	96.0	32
J. B.	9	M	TBC Meningitis	37	1.5	99.3	28
M. A.	9	M	Mumps	38	0.9	93.7	28
M. R.	10	M	Pityriasis rosea	41	1.0	96.4	33
J. G.	17	F	Guillain-Barré syndrome	42	1.3	93.1	30

DISCUSSION

Our data indicate that the half initial radioactivity time of Cr^{51}-labeled autogenous erythrocytes in children lies within the range reported for clinically healthy adults.[1-6, 13] This is compatible with the normal survival time of normal adult blood observed in "normal" children using the Ashby technique.[7-12] Kaplan and Zuelzer[8] reported a normal erythrocyte survival time for the erythrocytes of a child with "hypochromic microcytic anemia of the iron-deficiency type" in the circulation of another child. This appears to be the only previous report of erythrocyte survival time of a child's erythrocytes in another child.

The average 24-hour per cent of initial radioactivity in our series was 96.5 per cent. This is slightly greater than the 95 per cent reported by Donohue

and his co-workers[5] for unwashed cells and 94 per cent reported by Mollison and Veall[4] for washed cells. Our data do not permit us to identify the mechanism responsible for this initial loss.

SUMMARY

The half radioactivity time of chromium^{51}-labeled autogenous erythrocytes in 15 children from 2½ to 17 years in age, convalescing from infectious diseases, was found to be within the range reported as normal for adults.

REFERENCES

1. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F.: The Use of Radioactive Chromium^{51} as an Erythrocyte Tagging Agent for the Determination of Red Cell Survival In Vivo, J. Clin. Invest. **32**: 1260-1276, 1953.
2. Necheles, T. F., Weinstein, I. M., and LeRoy, G.: Radioactive Sodium Chromate for the Study of Survival of Red Blood Cells. I. The Effect of Radioactive Sodium Chromate on Red Cells, J. Lab. & Clin. Med. **42**: 358-367, 1953.
3. Sutherland, D. A., McCall, M. S., Groves, M., and Muirhead, E. E.: The Survival of Human Erythrocytes Estimated by Means of Cells Tagged With Radioactive Chromium: A Study of the Normal State, J. Lab. & Clin. Med. **43**: 717-726, 1954.
4. Mollison, P. L., and Veall, N.: The Use of the Isotope Chromium^{51} as a Label for Red Cells, Brit. J. Haemat. **1**: 62-74, 1955.
5. Donohue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Viranuvatti, V., and Finch, C. A.: The Use of Chromium as a Red-Cell Tag, Brit. J. Haemat. **1**: 249-263, 1955.
6. Hughes Jones, N. C., and Mollison, P. L.: The Interpretation of Measurements With Cr^{51}-Labeled Red Cells, Clin. Sc. **15**: 207-218, 1956.
7. Kaplan, E., and Zuelzer, W.: Erythrocyte Survival Studies in Childhood. I. Methods and General Observations, J. Lab. & Clin. Med. **36**: 511-516, 1950.
8. Kaplan, E., and Zuelzer, W.: Erythrocyte Survival Studies in Childhood. II. Studies in Mediterranean Anemia, J. Lab. & Clin. Med. **36**: 517-525, 1950.
9. Kaplan, E., and Zuelzer, W.: Erythrocyte Survival Studies in Childhood. III. Unusual Familial Hemolytic Anemias Associated With Intrinsic Erythrocyte Abnormality, J. Lab. & Clin. Med. **36**: 524-530, 1950.
10. Reinhold, J.: The Survival of Transfused Red Cells in Acute Rheumatic Fever With Reference to a Latent Hemolytic Mechanism, Arch. Dis. Childhood **29**: 201-212, 1954.
11. Berlin, R.: Red Cell Survival Studies in Normal and Leukemic Subjects, Acta med. scandinav. (Suppl. 252) **139**: 1-141, 1951.
12. Reinhold, J.: Personal Communication.
13. Remenchik, A. P., Dyniewicz, J. M., Schoenberger, J. A., and Osterberg, A. E.: Viability of Erythrocytes of Acid Citrate Dextrose Blood Collected and Stored in and Infused from Aged Plastic Bags, J. Lab. & Clin. Med. **48**: 469-470, 1956.

DEGRADATION OF IODINATED HUMAN SERUM ALBUMIN PREPARED BY VARIOUS PROCEDURES

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ALBUMIN labeled with I^{131}, in addition to its common use for blood volume determinations, has been used to estimate daily albumin synthesis and total body albumin stores in the normal individual.[1, 2] It has also been employed to study albumin turnover in patients with cirrhosis,[3] nephrosis,[4] hypothyroidism,[5] hyperthyroidism,[6] cancer,[7] regional enteritis,[8] and following such trauma as operations.[9] Transfer rates of albumin from plasma to ascitic fluid have also been estimated using I^{131}-labeled albumin.[10-12] In all of these studies in which measurements are made over many days the biologic equivalence of native serum albumin with I^{131} albumin has been assumed, although the dangers inherent in such an assumption have been pointed out repeatedly.[2, 13, 14]

Certain I^{131} albumin preparations have shown characteristics on electrophoresis and in the ultracentrifuge which are not distinguishable from native serum albumin,[1, 2] while other I^{131} albumin preparations differ from native albumin in electrophoretic[15, 16] or chromatographic behavior.[17]

In a previous study 3 different I^{131} albumin preparations were administered in different sequence to the same 9 individuals,[14] thus permitting a comparison of rates of degradation of different I^{131} albumins in the same individuals. The present collaborative study, an extension of the former, was undertaken in the hope of finding a commercially practical I^{131} albumin preparation for long-term metabolic studies.

Factors which may influence the biologic behavior of I^{131} albumin are: (1) the isolation procedure used for separating albumin from pooled plasma or serum; (2) the heating of albumin to 60° C. for 10 hours to minimize the possibility of homologous serum hepatitis transmission[18]; (3) the method of iodination (e.g., oxidizing agent, pH, and temperature)[13]; (4) the I^{127}-albumin molor ratio[2]; and (5) the radiation absorbed by the I^{131} albumin through beta and gamma emission during iodination of the albumin and storage of the I^{131} albumin.[19] The effects of varying factors 1, 2, and 3 above during the iodination procedure on the degradation rate of I^{131} albumin after intravenous administration to man are herein reported.

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MATERIALS AND METHODS

A. *Preparative Procedures.*—Three different commercial lots of Cutter human serum albumin (A, B, and C) were used for iodination in these experiments. All contained the usual preservatives and had been heated to 60° C. for 10 hours to minimize the possibility of homologous serum hepatitis transmission.

Lots A and C have been used repeatedly for the preparation of radioiodinated human serum albumin (RISA). There was nothing in the tests or apparent properties of lot B to suggest the abnormal results secured with it when used as an experimental raw material for this study. Albumin iodination was carried out at Abbott Laboratories under the supervision of one of the authors (D. L. T.)

1. *Iodination with I_2:* This was a modification of McFarlane's "jet iodination" technique[20] using, however, very rapid stirring rather than the special equipment which he has proposed and bicarbonate rather than a glycine buffer. Twenty millicuries of I^{131}, 0.04 mg. of KI, and 0.015 mg. of KIO_3 were rapidly mixed in bicarbonate buffer with 250 mg. of albumin, and after one hour the unbound iodide was separated on anion-exchange column. The iodine albumin molar ratio was 1:1 and the yield 2 to 4 per cent in terms of I^{131}.

2. *Iodine distillation method:* Because the presence of variable amounts of reducing agents in the sodium iodide-^{131}* interfered with the iodination reaction, preparations were made in which the iodide was distilled into a slightly basic solution, and the resultant iodide was used to iodinate the albumin. In a typical experiment 20 mc. of I^{131} and 0.4 mg. of iodine were incubated for 3 hours in the cold with 200 mg. of albumin in glycine buffer. The material was then passed over an ion exchange column to remove the nonbound iodine. The iodine-albumin molar ratio was 1:5 on the average, and the yield was variable but never exceeded 5 per cent.

3. *RISA:* Routine preparations of RISA from Abbott Laboratories were included in this study. In this preparation, a dilute solution of hypochlorite is added, with constant agitation and at intervals, to a mixture of I^{131}-labeled KI in the cold. After standing overnight, free iodide and iodate are removed on an ion exchange column; the material is diluted with further protein and normal saline and Seitz filtered for sterilization. The iodine-albumin ratio is 1:5.5 and the yield between 70 and 75 per cent.

4. *RISA T-1:* This material was similar in all respects to RISA except that either the number of millicuries of I^{131} was decreased or the reaction time was shortened so that the radiation absorbed by the albumin during manufacture was decreased to approximately 10,000 r.e.p.†

5. *RISA T-2:* This group of preparations was similar to T-1 above, except that the reaction was further modified so that the iodine-albumin molar ratio during iodination was 1:55 rather than 1:5.5. Probably because of the presence of more of the highly reducing sulfur compounds in the relatively larger quantity of albumin used in the iodination procedure the yields in terms of I^{131} were significantly lower.

6. *RISA T-3:* This was a modification of T-2 in which larger amounts of albumin were added after iodide and iodate were removed on an anion exchange column. This was done in an effort to decrease still further the effects which radiation from I^{131} following the iodination process itself might have upon the I^{131} albumin.

In each instance, the presence of unbound or loosely bound iodide or iodate was checked by paper chromatography, trichloroacetic acid protein precipitation, and dialysis experiments and found to be uniformly less (except one lot) than 1.0 per cent. Nine-tenths per cent of benzyl alcohol was added as a preservative, and supplies were stored in the refrigerator prior to use.

B. *Procedure of Study.*—Patients received Lugol's solution, 10 drops every 8 hours, for 2 days prior to the injection of the I^{131} albumin as well as during the entire period of study.

*Supplied by Carbide and Carbon Nuclear, Oak Ridge, Tenn.
†r.e.p. = roentgen equivalent physical.

Calibrated syringes were used for the injection of the I^{131} albumin which was introduced into the rubber tubing of a slowly running normal saline infusion. Three normal saline rinses were also injected into the patient before the infusion was stopped. Blood samples were drawn at 12 and 24 minutes for estimation of blood volume. Twenty-four-hour urine collections were obtained in bottles containing 10 ml. of a NaI, $NaHSO_3$, NaOH carrier solution.*

RESULTS

The majority of the studies to be reported were short-term (10 days) comparative excretion and metabolism studies using 20 μc of I^{131} albumin. In such studies more information about the albumin preparation than about the patient's albumin metabolism was obtained.

In addition, long-term (30 to 60 days) metabolic studies were carried out in control individuals and in patients with disease, using 100 μc of albumin I^{131}.

Tables I through VI present in detail the data obtained with the 6 different types of preparations.

TABLE I. I^{131} ALBUMIN (MODIFIED MCFARLANE TECHNIQUE)

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μc I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
1045-26	5-10-56	10,000	A	75.0	Pa.	8.9	4.5
					St.	9.1	6.6
					Ca.	6.6	3.0
					Ha.	6.6	3.4
					Sa.	8.0	2.9
					Be.	9.3	3.0
					Ar.	8.2	3.3
					Ka.	7.1	3.5
1045-37	7-31-56	12,000	B	4.0	Sc.	95	---
					Fu.	69	---
					Hy.	86	---
					Kr.	84	---
					Ha.	100	---

The next to the last column, entitled "Cumulative Per Cent Administered Radioactivity Excreted in 48 Hours," represents a summation of the excretion of I^{131} resulting from metabolism of presumably unaltered albumin proteins, plus that due to any protein which had been so denatured or modified as to be rapidly degraded. The final column gives the per cent per day degraded from days 7 through 10 inclusive. The per cent degraded each day was estimated by dividing the per cent of the administered radioactivity excreted on any day by the average per cent of the dose remaining in the body on that day. By the seventh day most of the rapidly degraded I^{131} albumin had disappeared, and the values as stated seemed to reflect the metabolic turnover of albumin for that patient under the conditions specified.

*Composition of carrier solution: 25 Gm. potassium iodide, 44 Gm. sodium bisulfite, and 80 Gm. sodium hydroxide in 1,000 ml. water. Stock solution is diluted 1:100 before use.

Preparation 1045-26 has previously been reported[8] and represents material with "good" biologic properties, in that less than 8 per cent of the administered radioactivity was excreted in the first 48 hours, and an average of 3.8 per cent of the iodinated albumin was degraded each day by 8 individuals from days 7 through 10. As indicated earlier, it was never again possible to prepare materials with these properties, even from lot A of the albumin.

The I^{131} from preparation 1045-37, made from lot B of albumin, was almost quantitatively excreted in 48 hours and gave plasma volumes which were as much as 10 times higher than expected. This method of preparation, plus some factor in albumin B, resulted in material totally unsuited for tests of this sort, or for other biologic experimentation.

TABLE II. I^{131} ALBUMIN (IODINE DISTILLED INTO ALBUMIN SOLUTION)

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μC I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
1045-57	10-18-56	15,000	A	33.0	Co.	24	4.1
					Du.	24	5.5
1045-63	10-29-56	13,000	A	33.0	Sh.	17	4.2
					Es.	18	2.9
					Ka.	15	3.8
1045-97	12-15-56	11,000	A	3.3	Mc.	58	---
					St.	63	---

These represent the preparations made by distilling iodine into an albumin solution. Yields were low and products could not be reproduced with assurance. Preparation 1045-97, though prepared from albumin A, was rapidly degraded and like 1045-37 gave abnormal blood volumes. Two other lots, 1045-57 and 1045-63, were not degraded differently than standard RISA preparations.

TABLE III. RISA

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μC I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
968-171-30	11-10-55	100,000	A	70	Cor.	16	5.4
					Wh.	16	5.0
					Cop.	21	6.4
					Gy.	17	6.1
					Ho.	18	4.8
968-267-2	2-10-56	150,000	A	70	We.	16	4.2
					Hy.	15	5.1
					El.	21	5.8

In Table III can be found results representative of studies in control individuals with most RISA preparations. Approximately 10 to 20 per cent of the injected radioactivity is excreted in the first 48 hours, and subsequently a gradually declining degradation rate is found. This rate usually stabilized by

the fifth to the eighth day as can be seen in Fig. 1, where an early, rapid degradation rate can be separated from a later slower component. That the presence of 10 to 20 per cent of rapidly degraded material does not alter the distribution curve is shown in the lower part of Fig. 1; it can be seen here that the per cent of the injected dose which is in the vascular compartment at equilibrium time compares favorably with other reported results.[2]

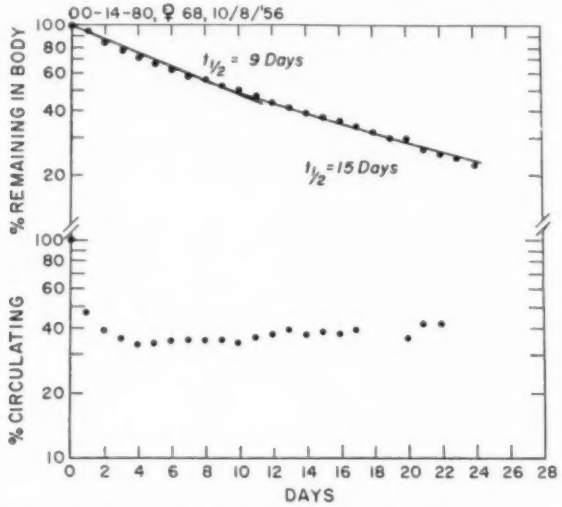

Fig. 1.—Degradation and distribution of I^{131} albumin (RISA). In the upper part of the figure on a semilogarithmic scale is plotted the per cent of the administered I^{131} albumin remaining in the body. Time in days is indicated on the abscissa.
In the lower part of the figure is plotted on the semilogarithmic scale the per cent of the I^{131} albumin remaining in the body which is intravascular.

TABLE IV. RISA T-1

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μC I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
1045-34	7-25-56	33,000	B	9.0	Ir.	33	4.5
					Mi.	33	4.3
					Ne.	33	3.8
					St.	32	4.3
1045-39	8- 3-56	47,000	B	14.0	Ir.	27	5.0
					Mi.	27	4.0
					Ne.	29	4.1
					St.	26	4.3
1045-56	10-18-56	6,700	A	15.0	Sh.	15	5.4
					Es.	14	4.2
					Ka.	13	4.0
1045-64	10-29-56	10,000	A	23.0	Co.	13	3.1
					Pu.	7	3.0

In Table IV can be found data obtained using a RISA preparation identical in all respects with the ordinary RISA except that iodination was carried

out in a more dilute solution of I^{131} and/or for a shorter period of iodination, thus decreasing the radiation dose to the albumin (RISA T-1). The per cent of the injected radioactivity excreted in the first 48 hours with preparation 1045-34 and 1045-39 was considerably greater than with the previous RISA preparations, despite the decreased radiation received by preparations 1045-34 and 1045-39. Preparations 1045-56 and 1045-64 were not excreted so rapidly though manufactured under identical laboratory conditions as 1045-34 and 1045-39. Lot B of commercial albumin was used in the first two preparations while lot A was used in the last two preparations, pointing to a fault in the albumin preparation even before iodination.

TABLE V. RISA T-2

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μC I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
1045-27	5-25-56	45,000	A	235.0	Av.	14	5.9
					Ka.	11	4.4
					Be.	10	5.9
					St.	10	2.6
					Ca.	11	3.2
					Pa.	12	4.2
					Ha.	11	4.2
					Sa.	14	3.3
1045-40	8-9-56	9,600	B	5.0	Se.	22	4.5
					Fy.	21	4.7
					Hay.	19	4.2
					Kr.	21	5.2
					Har.	21	4.7

TABLE VI. RISA T-3

PREPARATION	DATE ADMINISTERED	ALBUMIN SELF-RADIATION (R.E.P.)	STABLE ALBUMIN LOT	"SPECIFIC ACTIVITY" μC I^{131}/MG. ALBUMIN	PATIENT	CUMULATIVE % OF ADMINISTERED RADIOACTIVITY EXCRETED IN 48 HOURS	AVERAGE PER CENT IHSA DEGRADED (DAYS 7-10)
1045-110	1-11-57	10,000	A	2.0	Co.	11	2.4
					Lo.	11	4.3
					St.	16	5.9
1045-112	2-10-57	20,000	C	2.0	Je.	11	4.2
					My.	6	3.7
					Lo.	9	4.8
					Ry.	6	3.6
1045-114	3-15-57	20,000	C	2.0	Ro.	9	3.6
					Hu.	9	---
					Ur.	8	4.8
1045-116	4-11-57	20,000	C	2.0	Ah.	12	5.6
					Gh.	9	5.2
					Ne.	11	5.8
1045-118	5-22-57	9,000	B	2.0	Mu.	15	---
					Ev.	15	---
					Or.	18	---

Table V presents data on a preparation in which not only was radiation to the albumin decreased but also the iodine-albumin molar ratio was

decreased to 1:55.0 (RISA T-2). An average of 12 per cent of the radioactivity was excreted in the first 48 hours when lot A was iodinated, whereas 21 per cent was excreted in the first 48 hours when lot B was iodinated.

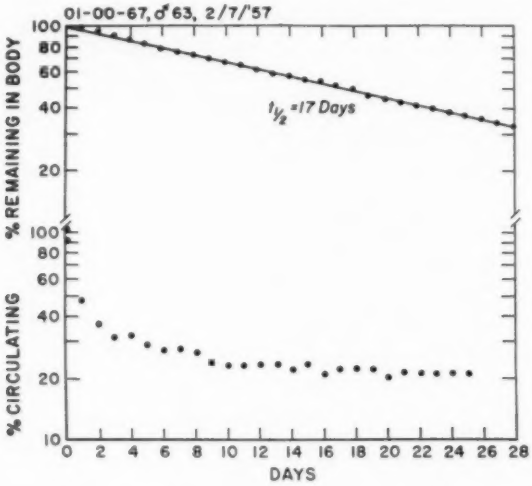

Fig. 2.—Degradation and distribution of I^{131} albumin (RISA T-3). In the upper part of the figure on a semilogarithmic scale is plotted the per cent of the administered I^{131} albumin remaining in the body. Time in days is indicated on the abscissa.

In the lower part of the figure is plotted on the semilogarithmic scale the per cent of the I^{131} albumin remaining in the body which is intravascular.

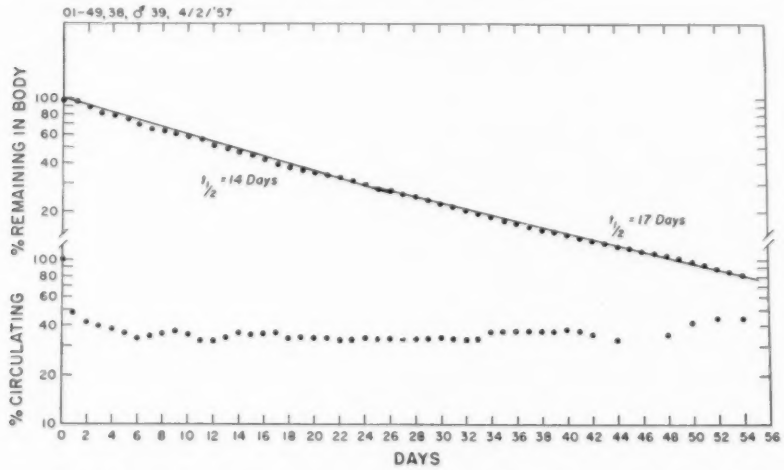

Fig. 3.—Degradation and distribution of I^{131} albumin (RISA T-3). In the upper part of the figure on a semilogarithmic scale is plotted the per cent of the administered I^{131} albumin remaining in the body. Time in days is indicated on the abscissa.

In the lower part of the figure is plotted on the semilogarithmic scale the per cent of the I^{131} albumin remaining in the body which is intravascular.

In the last of the preparations (RISA T-3), conditions for the preparation of RISA T-1 were employed (decreased albumin radiation), with the exception that large amounts of albumin rather than saline was used as a diluent. With albumin lot A an average of 13 per cent of the radioactivity was excreted in the first 48 hours; with lot C an average of 9 per cent was excreted in the first 48 hours; and with lot B an average of 16 per cent was excreted in the first 48 hours (Table VI).

The results of a typical 3 to 4 weeks' study following the intravenous administration to man of 100 μc of I^{131} albumin, RISA T-3, are shown in Fig. 2. In contrast with Fig. 1, a single line can be fitted to the graph of the log of the per cent of the radioactivity remaining in the body as compared with time. Since 100 μc of I^{131} is the usual dose used in such studies, the appearance of a single exponential in Fig. 2 indicates a uniform rate of degradation of the I^{131} albumin.

In Fig. 3 can be found the results of a study carried out on a patient who was suspected of having a brain tumor and who received 600 μc of I^{131} albumin prior to a gamma ray scan of the cranium. The study could be continued for 58 days before the counting rate of the urine dropped to levels too low for satisfactory measurement. In this instance, while a single exponential obtains for approximately 24 days, there is another slower component for the last 32 days.

DISCUSSION

Previous investigators have shown that when there are more than 10 iodine atoms per albumin molecule, the rate at which the body degrades I^{131}-labeled albumin is increased.[2] Also "strong" oxidizing agents like hydrogen peroxide,[13] if used with iodide to iodinate albumins, result in an I^{131} albumin which is more rapidly degraded than normal. Exposure of iodinated albumin in a concentration of 0.2 mg./ml. to 50,000 r.e.p. again increased the rate at which the iodinated albumin was degraded.[19] In the present investigation, another factor, the albumin preparation prior to iodination, has been found to affect the rapidity with which the I^{131} label is separated in vivo from albumin. Commercial lot B whether prepared as RISA T-1, RISA T-2, or RISA T-3 was more rapidly degraded during the first few days. It would also appear from Table VI that albumin lot C, when iodinated, provided a better biologic label than lot A in that the former was less rapidly degraded. RISA probably contains some I^{131} albumins which are rapidly degraded (Fig. 1) while RISA T-3 appears to have a uniform degradation rate (Fig. 2). Since most biologic studies are carried out with radioactivity doses as low as possible, data on I^{131} albumin degradation obtained from studies carried out for more than 3 to 4 weeks as in Fig. 3 are not readily available. The fact that there is a change in degradation rate of I^{131} albumin after approximately the third week and the fact that this change occurs in a patient who has received a preparation which presumably does not contain any rapidly degraded I^{131} albumins make tenuous any conclusions about absolute rates of albumin turnover derived

from data on I^{131} albumin. While a change as seen on day 24 in Fig. 3 may represent a real change in the patient's albumin metabolism, it is not at all possible to rule out the presence of several I^{131}-labeled albumins in the preparation administered.

However, comparative 30-day studies with I^{131} albumin in patients with disease may still give insight into relative rates of albumin metabolism if control studies are done concurrently with each I^{131} albumin tested.

Data on average degradation rate from days 7 through 10 for each of the preparations have been listed in the tables. Since individuals may differ considerably in the rate at which they degrade I^{131} albumin, comparison of preparations can be profitably made only in the same individual; however, it is of interest and of some importance to note that all preparations except 1045-37 (Table I) and 1045-97 (Table II) were degraded at rates of from 3 to 7 per cent per day after the first 6 days. Much of the data on albumin metabolism obtained with I^{131} albumin may be valid for comparison purposes since the studies usually were carried on for 3 to 4 weeks.

From the data in Table III through VI it would appear that preparations containing less and less rapidly degraded iodinated albumins are obtained as the albumin's radiation exposure is decreased and/or as the albumin concentration is increased.

1045-26 was the only one of 6 preparations made using nascent iodine as the iodinating agent which resulted in an I^{131} albumin with biologic properties comparable to RISA T-3 C. Failure to obtain consistently biologically "good" I^{131} albumin preparations using modifications of McFarlane's techniques must be attributed to technical factors; however, the reliable and satisfactory I^{131} yield with RISA T-3 preparations make the latter suitable for commercial production.

After albumin is isolated by the Cohn technique it is heated to 60° C. for 10 hours to minimize the possibility of transmission of homologous serum hepatitis. Recently, rabbit albumin heated to 56° C. for 10 hours before iodination has been shown to have a shorter biologic half-life than similarly iodinated unheated albumin.[16] That heat treatment is necessary in the case of human serum albumin has been shown by Murray who found hepatitis transmitted to volunteers by transfusions of 25.0 Gm. of unheated albumin but not by 10 transfusions of 25.0 Gm. of heat-treated (60° C. for 10 hours) albumin.[21, 22] A review of work in this field has been published recently.[23]

Except for preparations 1045-37 and 1045-97, all estimates of blood volumes were in the expected range. Blood volumes were estimated in 2 ways: (1) samples were taken at 12 and 24 minutes and the zero time extrapolation of the radioactivity per ml. of plasma (linear graph paper) was used to estimate plasma volume; and (2) the 12-minute plasma sample alone was used to estimate plasma volume. Using method 2, plasma volume was 80 ml. higher on the average, and in only one instance was it as much as 200 ml. higher. These results are in accord with those previously reported,[24] indicating that a single 12-minute sample is adequate for estimation of plasma volume in nonedematous subjects.

SUMMARY

1. I^{131}-labeled albumin preparations with a uniform degradation rate over an approximate 4-week period have been described. Such preparations had an iodine-albumin ratio of less than 1:5.5 and had received less than 20,000 r.e.p. before administration to man. The concentration of albumin was 50 mg./ml.

2. Different commercial albumin lots yielded I^{131}-labeled albumins with varying biologic properties following iodination. It cannot be assumed a priori that all batches of commercial albumin are identical.

3. Even though an I^{131}-labeled albumin preparation under investigation in man shows a uniform degradation rate for 4 weeks, it may, if such a study is continued for another 4 weeks, yield a slower degradation rate, thus making tenuous any conclusions about absolute values of albumin turnover from data derived with I^{131} albumin.

4. Although I^{131} albumin preparations may contain varying amounts of rapidly degraded I^{131} albumin (up to 20 per cent) the estimation of total exchangeable albumin and albumin turnover derived from data obtained from the second to fourth weeks of study may yield useful information for comparative purposes.

5. In these studies a single 12-minute blood sample was adequate for the estimation of blood volume in nonedematous individuals.

ADDENDUM

Since the above report was submitted for publication we have completed studies of another preparation, RISA T-4C, which differs from RISA T-3C in that the final radioactivity concentration in terms of I^{131} is approximately 250 μc/ml. (5.0 μc I^{131} per milligram of albumin). This preparation from lot C of Cutter albumin, on the basis of in vitro and in vivo studies as described in the body of the manuscript, cannot be distinguished from RISA T-3C and appears equally suitable for long-term studies in which iodinated albumin might be used.

REFERENCES

1. Sterling, K.: The Turnover Rate of Serum Albumin in Man as Measured by I^{131}-Tagged Albumin, J. Clin. Invest. **30:** 1228-1237, 1951.
2. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments With I^{131}-Labeled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32:** 746-768, 1953.
3. Sterling, K.: Serum Albumin Turnover in Laennec's Cirrhosis as Measured by I^{131}-Tagged Albumin, J. Clin. Invest. **30:** 1238-1242, 1951.
4. Gitlin, D., Janeway, C. A., and Farr, L. E.: Studies on the Metabolism of Plasma Proteins in the Nephrotic Syndrome, J. Clin. Invest. **35:** 44-56, 1956.
5. Schwartz, E.: The Effect of Thyroid Hormone Upon the Degradation Rate and Miscible Pool of Radioiodinated Human Serum Albumin in Myxedema, J. Lab. & Clin. Med. **45:** 340-347, 1955.
6. Lewallen, C. G., Rall, J. E., Berman, M., and Hamel, H.: The Effects of Thyroid Hormone on the Distribution Kinetics and Degradation of Iodo-Albumin, J. Clin. Invest. **34:** 949, 1955.
7. Steinfeld, J. L.: Hypoalbuminemia in Cancer Patients, Proc. Am. A. Cancer Research **2:** 149, 1956.
8. Steinfeld, J. L., Davidson, J. D., and Gordon, R. S.: A Mechanism for Hypoalbuminemia in Patients With Regional Enteritis and Ulcerative Colitis, J. Clin. Invest. **36:** 931, 1957.
9. Sterling, K., Lipsky, S. R., and Freedman, L. J.: Disappearance Curve of Intravenously Administered I^{131} HSA in Postoperative Injury Reaction, Metabolism **4:** 343-349, 1955.

10. Tyor, M., and Cayer, D.: The Rate of Disappearance of Iodinated Human Albumin From the Serum in Relation to Albumin Concentration, Total Circulating Albumin Content and the Presence of Ascites in Patients With Cirrhosis, J. LAB. & CLIN. MED. **39:** 874-881, 1952.
11. Berson, S. A., and Yalow, R. S.: The Distribution of I^{131} Labeled Human Serum Albumin Introduced Into Ascitic Fluid; Analysis of the Kinetics of a Three Compartment Catenary Transfer System in Man and Speculation on Possible Sites of Degradation, J. Clin. Invest. **33:** 337-387, 1954.
12. Schoenberger, J. A., Kroll, G., Eckert, E. L., and Kark, P. M.: Investigation of Transfer Rates of I^{131} Albumin in Ascites and Edema. II. Studies in Control Subjects and Patients With Cirrhosis, J. LAB. & CLIN. MED. **47:** 227-240, 1956.
13. Cohen, S., Halloway, R. C., Matthews, C., and MacFarlane, A. S.: Distribution and Elimination of I^{131} and C^{14}-Labelled Plasma Proteins in the Rabbit, Biochem. J. **62:** 143-156, 1956.
14. Steinfeld, J. L., Flick, A. L., Paton, R. R., Milch, R. A., Beach, F. E., and Tabern, D. L.: Distribution and Degradation of Human Serum Albumin Labeled With I^{131} by Different Techniques, Ann. New York Acad. Sc. **70:** 109-121, 1957.
15. Gabrieli, E., Goulian, D., Jr., Kinersly, T., and Collet, R.: Zone Paper Electrophoresis Studies on Radioiodinated Human Serum Albumin, J. Clin. Invest. **33:** 136-141, 1954.
16. Merchant, W. R., Masouredis, S. P., and Ellenbogen, E.: The Effects of Heat and Pasteurization on Albumin Preparations in Particular Reference to Radioisotope Labeled Materials, J. Clin. Invest. **36:** 914, 1957.
17. Fahey, J. L., and Steinfeld, J. L.: Chromatographic Difference Between Radioiodinated Albumin Preparations and Normal Human Serum Albumin, Proc. Soc. Exper. Biol. & Med. (In press.)
18. Minimum Requirements: Normal Serum Albumin (Human). U. S. Department of Health, Education, and Welfare, National Institutes of Health, 8th Revision, July, 1953.
19. Yalow, R. S., and Berson, S. A.: The Effect of Irradiation Damage of Albumin I^{131} on the Rate of Its in Vivo Metabolism With Special References to the Validity of Biologic Studies With I^{131}-Labelled Proteins, J. Clin. Invest. **35:** 746, 1956.
20. McFarlane, A. S.: Labelling of Plasma Proteins With Radioactive Iodine, Biochem. J. **62:** 135-143, 1956.
21. Murray, R.: Personal communication.
22. Murray, R., Diefenbach, W. C. L., Gellen, H., Leone, N. C., and Ratner, F.: The Problem of Reducing the Danger of Serum Hepatitis From Blood and Blood Products, New York J. Med. **55:** 1145-1150, 1955.
23. Murray, R.: Viral Hepatitis, Bull. New York Acad. Med. **31:** 341-358, 1955.
24. Pritchard, W., Moir, T., and MacIntyre, W. (With the Collaboration of Scott R. Inkley): Measurement of the Early Disappearance of Iodinated (I^{131}) Serum Albumin From Circulating Blood by a Continuous Recording Method, Circulation Research **3:** 19-23, 1955.

THE INTRACELLULAR LABELING OF EHRLICH MOUSE ASCITES TUMOR CELLS WITH RADIOCHROMATE

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DURING investigations on the therapy of tumors by the use of radioactive tumor antibody, we have had occasion to study the rate of elution of such antibody from the surface of Ehrlich mouse ascites tumor cells following subcutaneous implantation. Interpretation of results was complicated by lack of quantitative information on the disappearance or removal of antibody-coated cells from the inoculation site before a solid tumor had formed; that is, while the inoculated cells were still essentially in suspension. Such an occurrence, if active to a significant extent, would result in disappearance of radioactive antibody from the tumor site by means unrelated to elution from the implanted cells, and would demand the application of a suitable correction.

Gray and Sterling[1, 2] have used radiochromate to label erythrocytes, and subsequently Necheles, Weinstein, and LeRoy[3] have used radioactive sodium chromate in studies of erythrocyte survival under various conditions. Methods based on the intracellular labeling of erythrocytes are now used routinely in clinical studies. Since the problems encountered in our own work resolve themselves into the measurement of disappearance rates of tumor cells from the original inoculum, a method analogous to that used for erythrocytes has been tried and found to be applicable to the stable intracellular labeling of Ehrlich mouse ascites tumor cells, and is the subject of this report.

MATERIALS AND METHODS

Labeling of Tumor Cells.—Ascites fluid containing tumor cells was withdrawn from the peritoneal cavity of Swiss mice, and delivered into 10 ml. of Alsever's solution. The heavy suspension was then diluted to a concentration of 10×10^8 cells per 10 ml. volume, and radioactive sodium chromate added.* The mixture was rotated in a stoppered tube at the rate of 2 r.p.m. for 1 hour at room temperature, after which the cells were washed 3 times with Alsever's solution in a refrigerated centrifuge (35 g, 10 minutes, 0° C.). The washed cells were suspended in cold Alsever's solution and divided into 1 ml. aliquots containing 10×10^6 cells, in replicate tubes.

Per cent Radioactivity Uptake at Varying Chromate Concentrations.—The labeling procedure was carried out with a constant number of tumor cells, but using chromate amounts

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*Procured from the Radioactive Pharmaceuticals Division of Abbott Laboratories, Oak Ridge, Tenn., under the trade name Rachromate. The specific activity of the preparation was 43.4 mc. per gram $Na_2Cr^{51}O_4$.

of 0.6, 1.2, and 2.4 μg (10, 20, and 40 μc, respectively). After washing and resuspension, the total radioactivity was determined by counting in a well-type scintillation counter (Nuclear Chicago Model 192) and the percentage of total added radioactivity taken up by the cells calculated (Table I).

Partition of Radioactivity Following Cell Lysis.—One milliliter of labeled cell suspension containing 10×10^6 cells was subjected to sonic disintegration for 2 minutes in a Raytheon Sonic oscillator (Model DF-101). After lysis, particulate matter was sedimented by high speed centrifugation (10,000 g, 20 minutes) and the clear supernate decanted. The pellet was washed 3 times with 0.9 per cent sodium chloride, under the same conditions, and the original supernate was combined with the washings. Aliquots were counted for radioactivity as before. The pellet was resuspended in 5 ml. saline and the total suspension counted. Both sets of counts were compared with the total count on the original sample, made before lysis. All counts were corrected for background and decay. Results are presented in Table II.

Percentage Elution of Radioactivity In Vitro.—Replicate tubes, containing 10×10^6 labeled cells in 1 ml. Alsever's solution, were incubated at 5° C. over a 4-day period. At 24-hour intervals, tubes were withdrawn, the cells gently resuspended, and an estimate of the percentage of damaged cells (cells showing extrusion of intracellular contents) made by direct count in a Petroff-Hauser counting chamber; 0.01 ml. cell suspension was withdrawn for this purpose. After counting, the tubes were centrifuged to pack the tumor cells (35 g, 30 minutes, 0° C.). The conditions of centrifugation were selected to allow complete sedimentation without excessive cell compression. Aliquots of the supernates were removed, diluted to a volume of 2 ml., and counted for radioactivity, corrections being made for the amount withdrawn for cell counts. The percentage elution of radioactivity per day was determined and compared with the corresponding percentage damage, over the entire 4-day period (Table III and Fig. 1).

Effect of Labeling on the Ability of Tumor Cells to "Take" In Vivo.—In the course of our work with the Ehrlich ascites tumor, it has been found that, when subcutaneously inoculated, a minimum of 1×10^6 cells must be inoculated if it is desired to obtain 100 per cent "takes" at all sites inoculated. Ten Swiss mice were bilaterally inoculated in this way with normal untreated ascites tumor cells, and 10 more with freshly labeled cells carrying radioactivity to the extent of 5,600 counts per minute. The number of sites at which successful "takes" occurred were compared for the 2 groups after 7 days (Table IV).

TABLE I. PER CENT RADIOACTIVITY UPTAKE AT VARYING CHROMATE CONCENTRATIONS

AMOUNT RADIOCHROMATE ADDED			TAG ON 10×10^6 CELLS	PER CENT
μG	μC	C.P.M.	(C.P.M.)	UPTAKE
0.6	10	118,125	56,355	47.5
1.2	20	248,750	104,610	42.0
2.4	40	472,500	118,775	39.0

RESULTS

The results indicate that tumor cells, at least of the strain used, are indeed labeled by this method (Table I). The maximum percentage uptake obtained was approximately 47 per cent of the total added radioactivity, which is about one-half of the maximum uptake reported for erythrocytes.[3] While higher specific activity preparations can be made by increasing the amount of radiochromate added, there are two major disadvantages associated with chromate concentrations above a certain point. Thus, when 0.6 μg of $Na_2Cr^{51}O_4$ were used for labeling, a maximum uptake was obtained with minimum daily elution

of radioactivity (slightly above 1 per cent per day). The use of 1.2 and 2.4 μg of $Na_2Cr^{51}O_4$ resulted in progressively lower uptakes, and significantly increased daily elution (slightly above 2 and 3 per cent per day, respectively). The amount of cell damage encountered over the total 4-day incubation period does not differ significantly at the various chromate concentrations; in fact, experience shows that after identical incubation conditions, 80 to 90 per cent cell damage is frequently encountered with suspensions of tumor cells that have not been subjected to chromate treatment. Cells treated with 0.6, 1.2, and 2.4 μg of nonradioactive Na_2CrO_4 also are damaged to a substantially similar extent (Fig. 1 and Table III).

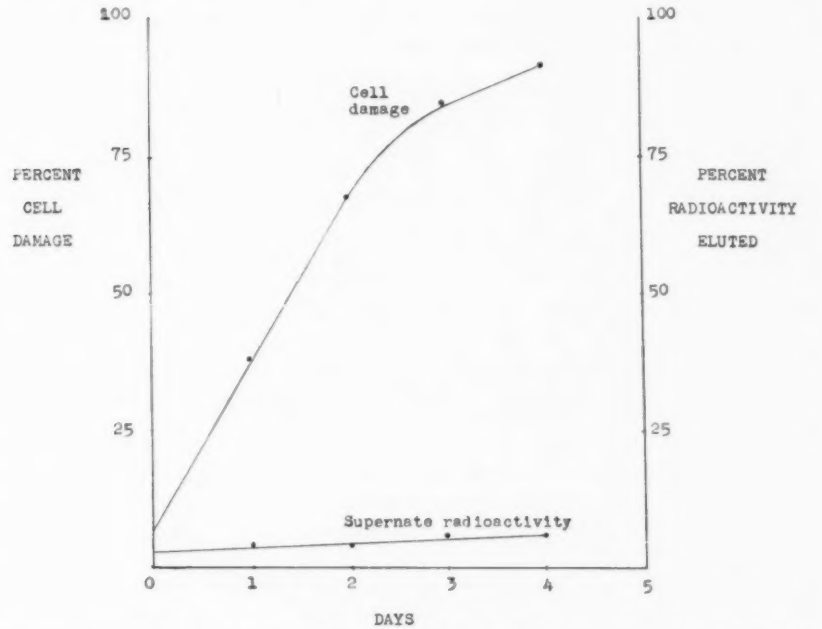

Fig. 1.—Percentage elution of radioactivity in vitro. (10×10^6 cells labeled at 0.6 μg level, 5° C.)

TABLE II. PARTITION OF RADIOACTIVITY FOLLOWING CELL LYSIS

	COUNTS PER MINUTE	PER CENT OF TOTAL
Total labeled cell suspension*	100,000	100.0
Particulate matter	9,600	9.6
Supernate	88,200	88.2

*Labeled cells, 1×10^6, in 1 ml. total volume.

It should be noted that when daily elution of radioactivity is studied at the most favorable chromate concentration (0.6 μg), no parallelism can be demonstrated between percentage cell damage and release of radioactivity into the supernate. This indicates that leakage from the cells, so long as they are intact,

is a very small contributing factor to elution of radioactivity. Thus, if the radioactivity can be shown to have a predominantly intracellular location, a very large percentage of released radioactivity must be due to actual cell rupture. An alternative interpretation is that considerable cell damage (without rupture) merely results in minimal elution.

TABLE III. PER CENT RADIOACTIVITY ELUTION* AT VARYING CHROMATE CONCENTRATIONS

AMOUNT RADIOCHROMATE ADDED (μg)	TAG ON 10×10^6 CELLS (C.P.M.)	CELL DAMAGE (PER CENT)	SUPERNATE RADIOACTIVITY (C.P.M.)	ELUTED CHROMATE (PER CENT)
0.6	56,355	89	3,332	5.9
1.2	104,610	88	11,169	10.7
2.4	188,775	81	22,825	12.1

*Values after 4 days; incubation at 5° C.

That the radioactive label is in fact intracellular is shown by the results of partition after cell lysis (Table II). Approximately 10 per cent of the total radioactivity is present in the particulate materials from the cells, while 88 per cent is present in the lysate. It has not proved possible to completely account for the remaining 2 per cent of the radioactivity except on the basis of loss during the several transfers during the procedure. The absolute proportion of intracellular radioactivity is certainly higher than indicated by these figures, since centrifugation sediments all particulate intracellular components. In spite of this, the particulate matter retains considerably less radioactivity than do the stroma of red cells (22 per cent).[3]

Animal experiments show that the ability of tumor cells, labeled with 0.6 μg $Na_2Cr^{51}O_4$ per 10×10^6 cells, to form solid tumors in mice is unimpaired (Table IV).

TABLE IV. EFFECT OF LABELING ON TUMOR "TAKE" IN VIVO

	NUMBER OF SITES INOCULATED	NUMBER OF POSITIVE "TAKES" (7 DAYS)
Normal untreated cells	20	20
Radiochromate* labeled cells	20	20

*Cells, 1×10^6, inoculated subcutaneously in 0.1 ml. volume.

DISCUSSION

In order that labeled cells may be used for survival or disappearance studies, the label used should satisfy the following criteria: it should have an intracellular location, should not leak out of the cells while the latter are alive, and should result in preparations sufficiently radioactive for in vivo tracer work. The results presented indicate that these criteria are essentially met by the radiochromate labeling procedure, which appears to be applicable to the Ehrlich ascites tumor cell as well as to the erythrocyte.

The partition studies of radiochromate distribution after cell lysis indicate that radioactivity is predominantly intracellular in location, the amount retained by cell particulates being even less than that reported retained by red cell stromata in similar experiments.

Since even when high percentages of the tumor cells are extruding intracellular material due to degeneration, only slightly more than 1 per cent per day of the radioactivity is released, it appears that leakage from the cells approaches the negligible in vitro, unless the cells are actually ruptured. It is to be noted that cell degeneration with accompanying extrusion of a portion of the intracellular material is not correlated with a corresponding rise in the radioactivity of the supernate, which indicates that, even when the cell has deteriorated to a major extent, only minimal amounts of radioactivity are released unless the cell has actually lysed. This supports belief in the stability of the label even under unfavorable conditions.

When labeled cells are used for studies in vivo, they are not in a resting state, but are presented with opportunities for multiplication. Since the in vitro results are, therefore, in no way comparable with possible occurrences in vivo, it would appear inadvisable to apply the correction we have determined in the test tube to animal experiments. A similar situation exists when erythrocytes are used for survival studies, but this does not diminish the usefulness of the technique in experiments conducted on a relative basis. The leakage from erythrocytes in vitro is essentially of the same magnitude as here reported for tumor cells.

It will be noted that in vitro studies on stability of the label have been carried out at low temperature. Results of experiments at 37° C. could not be interpreted for these studies in view of the massive deterioration, accompanied by considerable cell lysis, that occurred even when the samples were stored without disturbance at this temperature. There exists, in any event, a difference between in vivo and in vitro conditions too great to justify the view that incubation at 37° C. more closely parallels the physiologic situation.

The optimum amount of chromate (0.6 μg) used in labeling results in uptakes of the order of 55,000 counts per minute for 10×10^6 tumor cells, a level more than satisfactory for tracer experiments. It has also been shown that neither the radioactivity nor the chromate present in the cells affects their ability to "take" in animals when a minimal inoculum is used.

The intracellular location of the chromate ion in the red cell has been shown to be associated with the globin fraction.[3] It is not known, at this time, what the combination site may be in the tumor cell. This aspect is being studied at this time.

It is concluded that in all significant respects, there exists a parallelism between the labeling of erythrocytes and of Ehrlich ascites tumor cells with radiochromate. It is possible that other tumor cell strains can be labeled in this manner. The technique may also find use in tissue culture studies on cell multiplication, and in related areas.

SUMMARY

1. A method is described for the use of radioactive sodium chromate ($Na_2Cr^{51}O_4$) in the labeling of Ehrlich ascites tumor cells. The method is analogous to that used for labeling erythrocytes.

2. The label obtained by this method is largely intracellular, and elutes from the cells at the rate of slightly more than 1 per cent per day in vitro. Elution is not appreciable unless cell rupture occurs.

3. In the recommended doses, neither the chromate ion nor the radioactivity appears to injure the tumor cells either morphologically or in their ability to produce subcutaneous tumors.

4. The method is applicable to the study of the fate of cells after inoculation, and to tissue culture studies of growth characteristics of these and possibly other tumor cells.

REFERENCES

1. Gray, S. J., and Sterling, K.: Determination of Circulating Red Cell Volume by Radioactive Chromium, Science **112**: 179-180, 1950.
2. Gray, S. J., and Sterling, K.: Tagging of Red Cells and Plasma Proteins With Radioactive Chromium, J. Clin. Invest. **29**: 1604-1643, 1950.
3. Necheles, T. F., Weinstein, I. M., and LeRoy, G. V.: Radioactive Sodium Chromate for the Study of Red Blood Cells. I. The Effect of Radioactive Sodium Chromate on Red Cells, J. Lab. & Clin. Med. **42**: 358-367, 1953.

SERUM LACTIC DEHYDROGENASE AND GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITIES IN NORMAL PREGNANCY

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THE elevation of serum lactic acid dehydrogenase (LDH) activity has been noted in association with several diseases as well as normal pregnancy. Workers in this field have pointed out that "a broad survey of a large variety of disease states is required in order to evaluate properly the clinical significance of changes in serum LDH levels and the usefulness of such determinations as a diagnostic tool."[1] The elevation of serum LDH activity has been reported in pregnant women by Hill and Levi[2] and Hill.[3] The former studied 10 subjects; 7 of whom had LDH activities above normal. The latter's study consisted of 40 subjects; approximately half of whom had increased activity. MacDonald[4] observed only a minimal increase in serum LDH activity in 1 out of 5 pregnant subjects. No correlation of increased LDH activity was made to the period of gestation in any of the studies nor to our knowledge has serum glutamic oxalacetic transaminase (GOT) activity been studied in this regard.

The present experiments were undertaken to investigate the effect of normal pregnancy on serum LDH and GOT activities in relation to period of gestation, number of pregnancies, age, and body weight of the subject.

MATERIAL AND METHODS

Venous blood was drawn without regard to the fasting state from 100 pregnant women free of evidence of clinical disease and from 31 apparently healthy nonpregnant women of a similar age group (18 to 49 years). The blood was allowed to clot at room temperature for not longer than 30 minutes and then centrifuged.[1] The sera were frozen for not longer than 10 days if the determinations could not be run immediately.[5] Hemolyzed sera were rejected.

Lactic Dehydrogenase.—The two methods which are used for the assay of LDH are based upon the oxidation of reduced diphosphopyridine nucleotide (DPNH) or the reduction of diphosphonucleotide (DPN) using pyruvic or lactic acid, respectively.

$$\text{Pyruvic acid} + \text{DPNH} \underset{\longleftarrow}{\overset{\text{LDH}}{\longrightarrow}} \text{lactic acid} + \text{DPN}$$

The equilibrium of the reaction greatly favors pyruvate reduction; therefore, the former method was considered more ideal.[6] LDH activity was assayed by the method of Hill and

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Levi as modified by White.[7] A Beckman DU spectrophotometer was used for all measurements. The activity is expressed as units per 0.01 ml. of serum per 30 minutes. One unit equals a decrease in optical density of 0.001.

Glutamic Oxalacetic Transaminase.—GOT was assayed by the method of Karmen as modified by Hergt and Langin.[8] All assays were run at 25° C. using a Beckman DU spectrophotometer. One unit of GOT activity is equal to a decrease in optical density of 0.001 per ml. of serum per minute at 25° C.

RESULTS

The serum LDH activities of 31 healthy nonpregnant women ranged from 43 to 106 LDH units, with a mean of 74 ± 5. The 100 pregnant women exhibited serum activity from 42 to 119 LDH units with a mean of 75 ± 6 (Table I). The LDH activities of the pregnant and nonpregnant subjects were compared to the period of gestation, age, body weight (Table II), and number of pregnancies (Table III). These were not found to be significant variables. The serum GOT activities of 25 healthy nonpregnant women ranged from 7 to 31 GOT units with a mean of 16 ± 6. The serum activities in the 100 pregnant women ranged from 9 to 33 GOT units with a mean of 20 ± 5.

TABLE I. RESULTS OF STATISTICAL STUDY OF GOT AND LDH VALUES IN NONPREGNANT AND PREGNANT WOMEN

GROUP	NO. OF DETERMINATIONS	RANGE	MEAN
GOT			
Nonpregnant	25	7- 31	16 ± 6
Pregnant	100	7- 33	20 ± 5
LDH			
Nonpregnant	31	43-106	74 ± 5
Pregnant	100	42-119	75 ± 6

Numbers preceded by ± are standard deviations.

The "t" test of significance was applied to difference between means of the nonpregnant controls and the pregnant values. Probability for chance occurrence of this difference was > 0.20.

To evaluate the statistical significance of the GOT and LDH results the "t" test of significance[9] was applied to the difference between the means for the nonpregnant and pregnant values (Table I). The probability for chance occurrence of the difference in each case was > 0.20 indicating no statistically significant difference between the nonpregnant and pregnant groups.

DISCUSSION

According to the statistical study done on the results of the serum LDH activities determined on 100 normal pregnant subjects there was no statistically significant difference between the nonpregnant and pregnant groups. Tables II and III demonstrate that the increased LDH activities were minimal; furthermore in no case was there a significant correlation with the period of gestation or number of pregnancies. Age and body weight had no significant effect on the enzyme activity (Table II). The age range of the pregnant subjects was 18 to 42 years. Hill found higher activities in children, but stated

TABLE II. SERUM LDH ACTIVITY IN RELATION TO WEEKS OF GESTATION, AGE, AND BODY WEIGHT

GROUP	WEEKS OF GESTATION	NO. OF SUBJECTS	LDH UNITS* DISTRIBUTION < 106	LDH UNITS* DISTRIBUTION 107-119	LDH UNITS* RANGE	LDH UNITS* MEAN	LDH UNITS/AGE DISTRIBUTION < 5.72	LDH UNITS/AGE RANGE	LDH UNITS/AGE MEAN	LDH UNITS/BODY WEIGHT DISTRIBUTION < 0.99	LDH UNITS/BODY WEIGHT RANGE	LDH UNITS/BODY WEIGHT MEAN
Nonpregnant	0	31	31	0	43-106	74	31	1.17-5.72	2.99	31	0.31-0.99	0.61
Pregnant†	5	1	1	0	78-	78	1	3.55-	3.55	1	0.60-	0.60
	6	5	5	0	70- 86	81	5	1.89-3.65	2.93	5	0.50-0.71	0.63
	8	9	9	0	43- 77	62	9	1.71-3.85	2.39	9	0.35-0.65	0.48
	12	11	11	0	42-102	77	11	1.54-4.13	2.78	11	0.30-0.82	0.57
	16	8	7	1	45-115	69	8	1.13-4.60	2.68	8	0.28-0.84	0.49
	20	18	18	0	52- 97	71	18	1.33-4.04	2.69	18	0.42-0.94	0.55
	24	9	7	2	47-119	78	9	1.59-4.96	3.14	9	0.33-0.94	0.59
	28	10	10	0	44-103	77	10	1.84-4.68	3.25	10	0.28-0.75	0.63
	32	11	10	1	56-113	77	11	1.93-4.71	3.32	11	0.32-0.80	0.48
	36	10	7	3	59-118	87	10	1.84-5.13	3.74	10	0.41-0.74	0.60
	40	6	6	0	55- 86	72	6	2.07-3.74	2.63	6	0.31-0.61	0.49
	Labor	1	1	0	105-	105	1	4.38-	4.38	1	0.67-	0.67
	Delivery‡	1	1	0	47-	47	1	2.04-	2.04	1	0.32-	0.32
	Total	100	93	7	42-119	75	100	1.13-5.13	3.04	100	0.28-0.94	0.51

*Units per 0.01 ml. serum.
†Normal pregnancies.
‡Anesthetized.

that the adult range of activity "is approached at about 14 years of age."[10] Since our study contained no subjects under 14 years we could make no confirmation of this finding.

TABLE III. SERUM LDH ACTIVITY IN RELATION TO NUMBER OF PREGNANCIES*

GROUP	GRAVIDA	NO. OF SUBJECTS	LDH UNITS			
			DISTRIBUTION		RANGE	MEAN
			< 106	107-119		
Nonpregnant	–	31	31	0	43-106	74
Pregnant	1	25	23	2	52-115	73
	2	22	18	4	48-119	81
	3	20	20	0	42- 84	65
	4	12	11	1	54-118	74
	5	11	11	0	53-102	73
	6	9	9	0	45-102	74
	7	1	1	0	71-	71
Total		100	93	7	42-119	74

*Normal pregnancies.

MacDonald[4] measured LDH activity in 174 subjects. Five pregnant subjects were included in his study. Only one had an elevated LDH activity and this elevation was minimal.

Hsieh, Suntzeff, and Cowdry[11] studied LDH activity in pregnant mice. They reported normal values during pregnancy in mice. In principle these results are in consonance with those obtained in the present study.

After this research was completed a report on lactic dehydrogenase activity in pregnant women was published by Brody.[12] His study consisted of 10 normal pregnancies (7 weeks to term), 5 cases of abortion, and 5 cases of toxemias. In all of his 10 normal pregnant subjects the LDH activities were within normal range. It is of interest that his results on a much smaller group are analogous to the findings reported in this study.

GOT activities were determined on 100 pregnant women. Statistically there was no significant difference between the results in this group and those obtained in the nonpregnant group.

It has been shown that the determination of serum LDH and GOT activities may be used as adjuncts in the diagnosis of diseases such as myocardial infarction and viral hepatitis.[4, 13] The present investigation would indicate that LDH and GOT determinations may be relied on in the diagnosis of these disease conditions in pregnant subjects.

SUMMARY

Serum LDH and GOT activities were determined on 31 nonpregnant and 100 normal pregnant subjects.

There were no statistically significant differences in the results for LDH and GOT activities in the nonpregnant and pregnant groups.

Period of gestation, number of pregnancies, age, and body weight were not significant variables.

Normal pregnancy does not appear to influence serum LDH or GOT activities.

REFERENCES

1. Hsieh, K. M., and Blumenthal, H. T.: Serum Lactic Dehydrogenase Levels in Various Disease States, Soc. Exper. Biol. & Med. **91**: 626-630, 1956.
2. Hill, B. R., and Levi, C.: Elevation of a Serum Component in Neoplastic Disease, Cancer Res. **14**: 513-514, 1954.
3. Hill, J. H.: Serum Lactic Dehydrogenase in Cancer Patients, J. Nat. Cancer Institute **18**: 307-313, 1957.
4. MacDonald, R. P., Simpson, J. R., and Nossal, E.: Serum Lactic Dehydrogenase: A Diagnostic Aid in Myocardial Infarction, J. A. M. A. **165**: 35-40, 1957.
5. Wacker, W. E., Ulmer, D., and Vallee, B. L.: Metalloenzymes and Myocardial Infarction. II. Malic and Lactic Dehydrogenase Activities and Zinc Concentration in Serum, New England J. Med. **255**: 449-456, 1956.
6. Kornberg, A.: Lactic Dehydrogenase of Muscle. In Colowick, S. P., and Kaplan, N. O.: Methods in Enzymology. Preparation and Assay of Enzymes, New York, 1955, Academic Press, Inc., vol. 1, pp. 441-443.
7. White, L. P.: Serum Enzymes. I. Serum Lactic Dehydrogenase in Myocardial Infarction, New England J. Med. **255**: 984-988, 1956.
8. Hergt, K., and Langin, J. L.: Serum Transaminase Determination, Am. J. M. Sc. **233**: 69-76, 1957.
9. Chambers, E. G.: Statistical Calculations for Beginners, ed. 2, New York, 1952, Cambridge University Press.
10. Hill, B. R.: Some Properties of Lactic Dehydrogenase Activity in Human Blood Serum: Elevation in Neoplastic Disease, Proc. Am. A. Cancer Res. (Abst.) **2**: 24, 1955.
11. Hsieh, K., Suntzeff, V., and Cowdry, E. V.: Serum Lactic Dehydrogenase Activity as Indication of Neoplastic Growth and Regression, Proc. Soc. Exper. Biol. & Med. **89**: 627-629, 1955.
12. Brody, S.: Serum Lactic Dehydrogenase Activity During Human Pregnancy, Acta Endocrinol. **25**: 101-104, 1957.
13. Chinsky, M., Wolff, R. J., and Sherry, S.: Serum Transaminase Activity: A Comparison in the Pyruvic and Oxalacetic Transaminases, Am. J. M. Sc. **233**: 400-408, 1957.

PENETRATION OF HUMAN RED CELLS BY PENICILLIN, CHLORAMPHENICOL, AND TETRACYCLINE

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IN A number of infectious disease states intracellular parasitism plays an important part in the pathogenesis of infection. Apart from viral and rickettsial infections intracellular location of viable organisms is also a feature of certain bacterial diseases, in particular, tuberculosis, brucellosis, and typhoid fever. The importance of this host-parasite relationship has become more obvious following the introduction of specific forms of antibiotic therapy, since it has become apparent that the intracellular location of the organism may serve to protect it from the action of circulating antibiotic. However, the method of such protection is far from clear. It may be that in certain cases the antibiotic is unable to pass the cell wall barrier or it may be that the drug is inactivated by intracellular cytoplasmic components. It is also probable that different types of tissue cell vary in their ability to allow the passage of antibiotic agents through the cell wall.

The results presented here are concerned with the penetration of 3 antibiotics into one particular type of cell, namely, the human red cell. The red cell provides a convenient experimental model for this type of investigation though it is realized that the results are not necessarily applicable to other types of tissue cell.

MATERIALS AND METHODS

Stock Antibiotic Solutions.—These were prepared in sterile 0.85 per cent saline in the following concentrations: penicillin (crystalline penicillin G) 1,000 units per milliliter; chloramphenicol and tetracycline, each 1,000 μg per milliliter. Stock solutions were stored in the Deepfreeze without loss of potency during the period of investigation.

Test Antibiotic Solutions.—These were prepared on the day of test by dilution of the stock solutions in sterile 0.85 per cent saline to the required concentration.

Red Cells.—Fresh defibrinated blood was collected in 20 ml. amounts from donors in sterile Erlenmeyer flasks containing glass beads. The blood was centrifuged for 5 minutes at 3,000 r.p.m. The supernatant plasma and buffy coat cells were removed. The packed red cells were then treated with antibiotic solutions as described below.

Antibiotic Assays.—These were carried out in nutrient digest broth* using serial doubling dilutions of the test substance in 0.5 ml. amounts. The test organism employed was *Staphylococcus pyogenes* var. *aureus* (N.C.T.C.—6571). The inoculum consisted of an 0.01 ml. volume of an 8-hour digest broth culture. Assay tubes were incubated for 24 hours and

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the presence or absence of growth was checked by making Gram stained films and by subculturing onto blood agar plates which were incubated for a further 24 hours. Antibiotic standard solutions were included in each set of assays.

RESULTS

The following experimental procedures were carried out.

Penetration of Red Cells by Penicillin.—This experiment was designed to determine whether penicillin would penetrate normal human red cells. One milliliter quantities of penicillin solution (200 units per milliliter) were added to aliquot amounts of red cells in 8 sterile 3 by ½ inch tubes. The tubes were then incubated at 37.0° C. with continuous gentle shaking for periods of 15 minutes to 6 hours. The tubes were centrifuged at 3,000 r.p.m. for five minutes and the supernatant was removed. The cells were then transferred to sterile universal containers and washed 3 times with sterile 0.85 per cent saline, in 25 ml. amounts. Two milliliter quantities of the final wash were retained for antibiotic assay. To the deposited packed cells was added 0.5 ml. volumes of sterile distilled water and hemolysis was assisted by repeated freeze-thawing. The disrupted cell suspensions were assayed for antibiotic content. Control cells treated as above, except for the addition of penicillin, were included. Results of a typical experiment are detailed in Table I, which shows the content of penicillin after 3 hours' incubation of cells plus antibiotic.

TABLE I. PENICILLIN IN RED CELLS (3 HOURS' INCUBATION)

	TUBES								
	1	2	3	4	5	6	7	8	9
Antibiotic treated cells	–	–	–	–	–	+	+	+	+
Control cells	+	+	+	+	+	+	+	+	+
Final saline wash	+	+	+	+	+	+	+	+	+

+ = Growth. – = No growth.
Concentration of penicillin in Tube 1 (Dil. ½) approximately 5 units per milliliter, i.e., approximately 10 per cent of the extracellular fluid concentration.

The concentration of penicillin in the cells was approximately 10 per cent of that in the extracellular fluid. However, the initial concentration in the cells may have been higher since a certain amount of loss may take place owing to diffusion into the washing fluid. Any such loss is probably of small amount since repeated washing of the cells in saline does not appreciably reduce the cell antibiotic content. In addition, the results reflect the presence of active penicillin only and do not allow for antibiotic which might conceivably be bound to cytoplasmic components and thus be inactivated.

The slope of the curve obtained by plotting penicillin cell content against time of incubation of cells with antibiotic rises steeply, maximal concentration being obtained in the first 30 minutes. Serial samples assayed at intervals of incubation between 30 minutes and 6 hours did not appear to contain additional amounts of penicillin.

The Effect of Plasma on Penicillin Uptake by Red Cells.—It was thought that the penetration of red cells by penicillin might in some way be determined

by the presence of a plasma component which might allow for adsorption of the molecule to the cell surface. To investigate this point the above experiment was repeated using 2 aliquot portions of red cells. To the one was added penicillin dissolved in the homologous plasma and to the other penicillin in saline. Assay results are shown in Table II.

TABLE II. EFFECT OF PLASMA ON PENICILLIN UPTAKE BY RED CELLS (2 HOURS' INCUBATION)

	TUBES							
	1	2	3	4	5	6	7	8
Antibiotic in plasma + cells	–	–	–	–	–	+	+	+
Antibiotic in saline + cells	–	–	–	–	–	+	+	+
Control cells	+	+	+	+	+	+	+	+

+ = Growth. – = No growth.

The results detailed in Table II would seem to indicate that the cell uptake of penicillin is independent of the presence of any plasma factor and that the antibiotic can pass directly into the cell.

Distribution of Penicillin Within the Red Cell.—It seemed likely that if penicillin were adsorbed firmly to the red cell surface then it might not be possible to remove it by washing in a physiologic salt solution. In this event subsequent assay might lead to the conclusion that the antibiotic was intracellular whereas its distribution in the cell might well be confined to the external cell surface. The following procedure seemed to resolve this point to some extent.

One and one-half milliliter aliquot volumes of red cells and penicillin solution (final concentration, 400 units per milliliter) were incubated at 37.0° C. for 2 hours. The cells were then washed with saline as described. Twenty milliliters of sterile distilled water was added to the deposited cells and after 20 minutes at room temperature the hemolyzed blood was centrifuged at 5,000 r.p.m. for 30 minutes. The supernatant hemoglobin solution was removed and retained for assay. The deposited cell "ghost" membranes were then washed twice in 20 ml. of distilled water to free them from hemoglobin as far as possible. The membranes were then assayed for antibiotic content. Results are shown in Table III.

TABLE III. DISTRIBUTION OF PENICILLIN BETWEEN CELL MEMBRANES AND HEMOGLOBIN

	TUBES									
	1	2	3	4	5	6	7	8	9	10
Cell membranes	+	+	+	+	+	+	+	+	+	+
Hemoglobin fraction	–	–	–	–	–	–	–	+	+	+

+ = Growth. – = No growth.

Table III shows that no penicillin was detected on the cell membranes and that growth occurred in the first tube. This does not imply that penicillin was not adsorbed to the cell membrane before washing since it may have been eluted into the washing fluid, but such a combination, if it exists, must be of loose type. The hemoglobin solution, on the other hand, contained appreciable amounts of penicillin approximately to a concentration of 10 per cent of the initial extracellular concentration suggesting that most, if not all,

of the penicillin exists in the hemoglobin fraction. Essentially similar results were obtained with rabbit cells. These were employed since the "ghosts" of rabbit red cells are more easily rendered free of hemoglobin than is the case with human red cells. In order to determine the distribution of penicillin within the hemoglobin fraction the above experiment was repeated and the hemoglobin antibiotic containing solution was dialyzed against running tap water for 36 hours. Subsequent assay of the solution and of an aliquot from the same sample which had not been dialyzed failed to demonstrate any penicillin in the former (Table IV).

TABLE IV. PENICILLIN IN HEMOGLOBIN FRACTION BEFORE AND AFTER DIALYSIS

	TUBES									
	1	2	3	4	5	6	7	8	9	10
Fraction 1 (nondialyzed)	–	–	–	–	–	–	–	+	+	+
Fraction 2 (dialyzed)	+	+	+	+	+	+	+	+	+	+

+ = Growth. – = No growth.

These findings suggest that penicillin exists in the hemoglobin fraction in a state of simple solution and is not bound to any specific part of the hemoglobin molecule.

Penetration of Red Cells by Chloramphenicol.—The experimental procedure involved was similar to that in the case of penicillin. Final chloramphenicol concentrations tested ranged from 25 to 250 μg per milliliter. The results of a typical experiment are shown in Table V.

TABLE V. CHLORAMPHENICOL IN RED CELLS (3 HOURS' INCUBATION)

	TUBES					
	1	2	3	4	5	6
Chloramphenicol + cells (25 μg/ml.)	+	+	+	+	+	+
Chloramphenicol + cells (100 μg/ml.)	+	+	+	+	+	+
Chloramphenicol + cells (250 μg/ml.)	+	+	+	+	+	+
Control cells	+	+	+	+	+	+

+ = Growth.

Repeated attempts with this technique failed to demonstrate the presence of chloramphenicol within the red cell. The antibiotic appears to be loosely adsorbed to the cell surface and most of it is removed in the early washings. Red cells suspended in saline have a negative electrostatic charge whereas the chloramphenicol molecule is positively charged in virtue of its primary amino group. The resultant effect would be to allow for adsorption of the drug to the cell surface, but the adsorption does not appear to be of firm type. Increasing the period of incubation up to 6 hours did not affect the results.

Effects of Chloramphenicol on Trypsinized Red Cells.—The above experiment was repeated using fresh trypsinized cells in place of normal cells. These were prepared according to the method of Morton and Pickles.[1] Assay results again failed to show chloramphenicol within the cells either attached to the membranes or within the hemoglobin fraction. This is perhaps not unexpected

since trypsin will not affect the lipoid structure of the cell wall and it is possible that the high lipoid content of the red cell wall is the main barrier to the passage of antibiotics.

Penetration of Red Cells by Tetracycline.—Red cells were treated as before with varying concentrations of tetracycline. Table VI shows the results obtained following an incubation period of 3 hours.

TABLE VI. TETRACYCLINE IN RED CELLS (3 HOURS' INCUBATION)

	TUBES									
	1	2	3	4	5	6	7	8	9	10
Tetracycline + cells (125 μg/ml.)	–	–	–	–	–	–	–	+	+	+
Tetracycline + cells (12.5 μg/ml.)	–	–	–	–	+	+	+	+	+	+
Tetracycline + cells (1.25 μg/ml.)	–	+	+	+	+	+	+	+	+	+
Control cells	+	+	+	+	+	+	+	+	+	+

+ = Growth. – = No growth.

Concentration in original washed cells approximately 45 to 50 per cent of that in the extracellular fluid.

Tetracycline appears to be able to pass the cell wall barrier to a greater concentration than penicillin. Varying the period of initial incubation between 30 minutes and 6 hours did not appreciably alter the final result, and the slope of the curve obtained by plotting cell concentration of antibiotic against time of incubation was similar to that for penicillin. Approximately 45 to 50 per cent of the extracellular concentration was found in the cells. Here again the antibiotic appeared to be in simple solution in the hemoglobin fraction.

DISCUSSION

The results detailed above show that there are apparent differences in the ability of various antibiotics to pass the red cell wall barrier. In the case of penicillin the concentration was about 10 per cent of that in the extracellular fluid, while the concentration of tetracycline was of the order of 45 to 50 per cent. Rammelkamp and Keefer[2] found a red cell concentration of penicillin of about 8 per cent. In the case of chloramphenicol no evidence of passage into the red cell was obtained. Glazko, Wolf, and Dill[3] found that chloramphenicol appears to be attached to the red cell surface by adsorption but they also failed to demonstrate it within the cell. With regard to the penetration of other types of cells by chloramphenicol, Smith, Worrel, and Swanson[4] have shown that it inhibits the esterases of bacteria and crystalline liver esterase, but that the esterase of animal mitochondria is not so inhibited suggesting that there is a barrier to the passage of the antibiotic at the cell wall.

We have not attempted to discuss the kinetics of the passage of antibiotic into the red cell. This is at present under investigation in the case of penicillin. However, it would seem that in view of the differences in results obtained with penicillin and chloramphenicol it is probable that additional factors are concerned other than mere diffusion through the membrane due to differences in molecular size. Both chloramphenicol and penicillin have small molecules (323 and 356, respectively). The electrostatic forces which may operate at the

red cell surface in the case of chloramphenicol may result in fixation of the molecule at the cell surface and prevent its further passage into the cell. It may also be that combination of the antibiotic molecule with some chemical group on the cell surface is a necessary preliminary step before it can pass the cell wall barrier, but there is no direct evidence that this is so.

An alternative explanation can be offered for the failure to demonstrate chloramphenicol within the red cell; namely, that inactivation may take place due to binding to a cellular component. It is known, for example, that antimicrobial activity may be inhibited in tissue by substances such as nucleic acids, keto acids, pyridoxal, and para-aminobenzoic acid (Dubos[5]).

It has been pointed out that results obtained with an experimental model of the red cell type are not necessarily applicable to other cells. It is known that some antibiotics may exert a specific action on certain types of cells but not on others. Gramicidin, for example, is toxic to red cells and to spermatozoa, producing lysis of the former, but has no effect on other tissue cells. These observations suggest that the selective action may be due to differences in the ability of the antibiotic to pass the cell wall barrier or to be adsorbed to cell surface receptor sites, if such a mechanism exists. Such differences are presumably a reflection of the chemical structure of the cell membrane. However, we have investigated the penetration of normal buffy coat white cells, leukemic white cells, and guinea pig peritoneal exudate cells by chloramphenicol and penicillin and have obtained results more or less similar to those obtained with red cells.

A limited number of investigations have been carried out to determine the effect of antibiotics on viable intracellular organisms. Mackaness[6] studied the action of various antibiotics on tubercle bacilli contained within monocytes in vitro. He found that streptomycin could inhibit the growth of such organisms but that very much higher concentrations were required in the extracellular fluid to inhibit the intracellular organisms than were required to inhibit the same organisms outside the cells. Suter[7] reported somewhat similar findings with streptomycin and tubercle bacilli. Magoffin and Spink[8] on the other hand, found that phagocytosed forms of brucella organisms were protected from the effect of streptomycin. Our own findings with phagocytosed forms of *Salmonella typhi* in vitro also seemed to indicate protection from the effect of streptomycin (Watson[9]). These results are not necessarily contradictory since the effect of streptomycin will depend on (1) the type of organism, (2) the concentration of antibiotic in the extracellular fluid, and (3) the state of the organism within the phagocytic cell, i.e., whether or not it exists in a phagocytic vacuole. Mackaness[6] also found that both Terramycin and viomycin were able to inhibit intracellular tubercle bacilli but that para-aminosalicylic acid had no such effect. Mackaness noted that Terramycin was equally active against intracellular tubercle bacilli and bacilli grown in Dubos' medium. This lends support to the view that drugs containing the basic tetracycline group are able to penetrate the cell wall barrier more readily than others.

Results obtained with in vitro type experiments using phagocytosed organisms may have little relation to events occurring in vivo since the biochemical environments in the two cases may be quite different. It is known, for example, that explants from splenic tissue of animals infected with tubercle bacilli in vivo are soon overgrown by the organisms in in vitro culture whereas this effect in vivo is not apparent. It is also known that a lowering of the oxidation-reduction potential of tissue may inhibit the growth of tubercle bacilli. Such observations may be indicative of changes in metabolic processes taking place at the cell wall and consequently the ability of an antibiotic to inhibit an intracellular organism in vitro may not reflect its in vivo action. Again, if an antibiotic passes into a cell in active form it does not follow that intracellular organisms will be affected by its presence. In the case of phagocytosed forms of *S. typhi,* for example, the organisms appear to be located in a vacuole within the cell. These vacuoles would appear to possess some form of limiting membrane which may hinder antibiotic passage into them. With other phagocytosed organisms, however, such as the tubercle bacillus, it appears that they lie free in the cell cytoplasm and this may be part of the explanation for the differences observed between tubercle bacilli on one hand and brucella organisms and *S. typhi* on the other, with regard to the action of streptomycin. Another factor which may affect the action of intracellular antibiotic is the pH of the phagocytic vacuole. It has been known for many years that phagocytosis of certain substances is followed by a marked fall in the pH of the phagocytic vacuole. If similar pH changes accompany the phagocytosis of certain bacteria there may well be inactivation of antibiotics such as streptomycin whose optimum activity is on the alkaline side.

One of our main interests relating to the effect of antibiotics on intracellular parasites has been concerned with the action of chloramphenicol on phagocytosed forms of *S. typhi.* In particular we have been interested in the role which these organisms may play in initiating the relapse phase of illness (Watson[10]). The results reported here support to some extent the view that these organisms may be protected from circulating chloramphenicol and are potentially available to bring about clinical relapse.

SUMMARY

The ability of 3 antibiotics, penicillin, chloramphenicol, and tetracycline, to penetrate human red cells was investigated. Penicillin appears to be able to penetrate the cell wall to approximately 10 per cent of the extracellular concentration, and the distribution of the antibiotic within the cell appears to be in the hemoglobin solution in a state of simple solution. Tetracycline appears to pass into the red cell to a greater extent than penicillin, but chloramphenicol was found to be unable to pass the cell wall barrier in active form in the concentrations employed.

The relationship of these findings to some of the problems of intracellular parasitism is discussed.

REFERENCES

1. Morton, J. A., and Pickles, M. M.: The Proteolytic Enzyme Test for Detecting Incomplete Antibodies, J. Clin Path. **4**: 189, 1951.
2. Rammelkamp, C. H., and Keefer, C. S.: The Absorption, Excretion and Distribution of Penicillin, J. Clin. Invest. **22**: 425, 1943.
3. Glazko, A. J., Wolf, L. M., and Dill, W. A.: Distribution of Chloramphenicol (Chloromycetin) and Its Metabolic Products Between Human Red Cells and Plasma, Proc. Soc. Exper. Biol. & Med. **72**: 602, 1949.
4. Smith, G. N., Worrel, C. S., and Swanson, A. L.: Inhibition of Bacterial Esterases by Chloramphenicol (Chloromycetin), J. Bact. **58**: 803, 1949.
5. Dubos, R. J.: Biochemical Determinants of Microbial Diseases, Cambridge, 1954, Harvard University Press.
6. Mackaness, G. B.: The Action of Drugs on Intracellular Tubercle Bacilli, J. Path. Bact. **64**: 429, 1952.
7. Suter, E.: The Multiplication of Tubercle Bacilli Within Normal Phagocytes in Tissue Culture, J. Exper. Med. **96**: 137, 1952.
8. Magoffin, R. L., and Spink, W. W.: The Protection of Intracellular Brucella Against Streptomycin Alone and in Combination With Other Antibiotics, J. LAB. & CLIN. MED. **37**: 924, 1951.
9. Watson, K. C.: The Effect of Chloramphenicol on the Isolation of Salmonella typhi From the Blood Stream, J. Clin. Path. **8**: 55, 1954.
10. Watson, K. C.: The Relapse State in Typhoid Fever Treated With Chloramphenicol, Am. J. Trop. Med. & Hyg. **6**: 72, 1957.

COMPARATIVE EFFECTS OF ADRENAL ENUCLEATION AND ADRENAL PEDICLE LIGATION ON BLOOD PRESSURE IN RATS

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ADRENAL enucleation with contralateral adrenalectomy elicits hypertensive disease in rats.[1] During a recent study we reviewed other adrenal manipulations which have been described as causing hypertension and, among these, selected ligation of the adrenal vascular pedicle with contralateral adrenalectomy as most likely to have the effects of adrenal enucleation.[2] This procedure had been described by Poumeau-Delille and associates,[3] who applied it to rats of widely varying size and noted, in passing, the occurrence of hypertension in a few of them.

The present report describes the effects of this procedure on blood pressure and the vasculature of young rats; these have been shown to be more susceptible to the hypertensive effect of adrenal regeneration than older rats.[4] We also describe below the effects of excess salt on the process and further compare the course of this hypertension with that provoked by adrenal enucleation.

METHODS

In a first experimental series, Sprague Dawley male rats weighing 90 to 110 grams were divided into 4 groups: adrenal enucleation was performed in Groups I and II and adrenal pedicle ligation in Groups III and IV. These two surgical procedures were associated with uninephrectomy and uniadrenalectomy. After the operations, all the rats were given 1 per cent saline to drink in order to counteract the effects of the transient postoperative adrenal insufficiency. On the tenth day, saline was replaced by tap water in Groups I and III. Blood pressure was taken once a week.[5] The animals were sacrificed on the seventh postoperative week; tissues were fixed in Susa's fluid, weighed, and examined microscopically following staining with periodic acid fuchsin.

In a second experimental series, 24 rats weighing 90 to 115 grams were divided into 2 groups in which the adrenals were either enucleated or ligated. The techniques of adrenal pedicle ligation in the two experimental series were slightly different. In the first series, the adrenal was gently pulled out and a ligature placed around the vascular pedicle and the surrounding periadrenal fat. In the second series, most of the periadrenal fat was dissected away exposing the vascular pedicle which was ligated. All the rats had 1 per cent saline to drink. On the sixtieth day when chronic hypertension was established,

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attempts were made to reverse the process by substituting water for saline or adding hydralazine* to the drinking water at the dose level of 5 mg. per 100 ml. of fluid. The animals were sacrificed one month later and autopsied as above.

RESULTS

First Experimental Series.—Rats subjected to adrenal enucleation and those with adrenal pedicle ligation differed somewhat during the first postoperative week in that adrenal insufficiency, which was present in both groups, was more severe in those with ligated adrenal pedicles. This was indicated by an initial loss of weight, delayed and slow growth, delayed increase in blood pressure, and greater initial mortality in rats with ligated pedicles.

This insufficiency, with its increased susceptibility to stress, such as that involved in measuring arterial pressure, delayed our making these measurements and also delayed replacement of saline with tap water in Groups I and III. As to mortality, there were only 2 deaths out of 21 rats with adrenal enucleation during the first 9 days of the experiment, while 9 died in a like group after pedicle ligation.

TABLE I. PRESSOR AND MORPHOLOGIC EFFECTS OF ADRENAL ENUCLEATION AND ADRENAL PEDICLE LIGATION

GROUPS AND TREATMENT	PER CENT OF RATS WITH BLOOD PRESSURE OVER		HEART WEIGHT* (GM.)	KIDNEY WEIGHT* (GM.)
	150 MM. Hg	200 MM. Hg		
I. Adrenal enucleation plus water	33	0	0.32 (0.25 to 0.48)	0.65 (0.56 to 0.95)
II. Adrenal enucleation plus saline	100	80	0.45 (0.35 to 0.67)	1.04 (0.66 to 1.43)
III. Adrenal pedicle ligation plus water	40	0	0.28 (0.25 to 0.32)	0.63 (0.55 to 0.73)
IV. Adrenal pedicle ligation plus saline	100	50	0.34 (0.27 to 0.43)	0.73 (0.68 to 0.81)

*Expressed in per cent of body weight.

Data from rats surviving more than 9 days are presented in Table I. These show that adrenal pedicle ligation has effects on blood pressure, heart weight, and kidney weight similar to those elicited by adrenal enucleation and that the hypertension is more common and severe when salt water is substituted for tap water. It should be noted that provision of water instead of salt did not wholly prevent the development of hypertension from either enucleation or ligation. At autopsy, the gross appearances of the organs and vessels of those animals which received salt were similar to those previously described[6] in hypertensive animals, with cardiac enlargement and scarring, kidney enlargement, and nephrosclerosis, and mesenteric arteritis; no gross abnormalities were evident in animals given tap water.

The microscopic lesion in heart, kidney, and mesenteric vessels were severe and similar in the salt-treated rats (Groups II and IV) (Fig. 1). Slight but definite lesions were also present in the rats given tap water (Groups I and

*Apresoline; Ciba Pharmaceutical Products, Inc., Summit, N. J.

III), but they were limited to the kidney and consisted of focal fibrinoid necrosis of the glomerular tuft, thickening of glomerular and tubular membranes, and occasional casts (Fig. 1); they were found only in those rats given tap water that developed blood pressures as high as 170 mm. Hg. No medullary tissue could be detected in the adrenals of either the enucleated or pedicle-ligated groups; the regenerated adrenal cortical tissue seemed morphologically

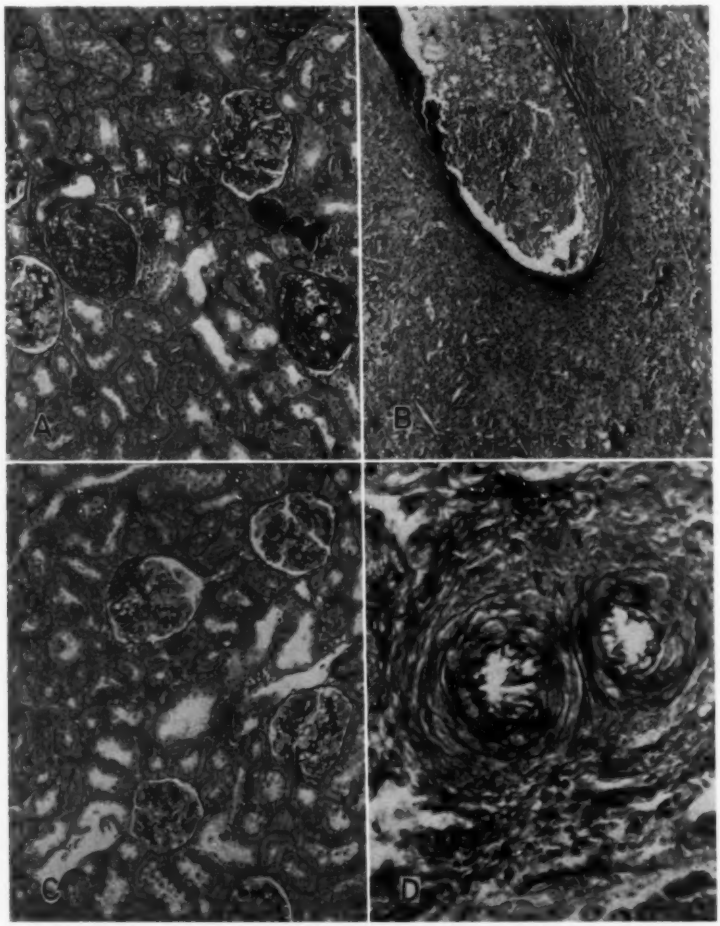

Fig. 1.—Tissues from rats with ligated adrenal pedicles that received 1 per cent saline (A and B), tap water (C), or saline followed by tap water (D) as drinking fluid. PAS stain. *A*, Kidney showing hyalinization of glomeruli, thickening of glomerular and tubular membranes, atrophy and dilatation of tubules, and hyalin casts. (Magnification ×130; reduced 2/7.) *B*, Mesenteric artery showing acute panarteritis. (Magnification ×165; reduced 2/7.) *C*, Kidney showing focal fibrinoid necrosis with synechiae in glomeruli, enlarged tubules, and thickening of basement membranes. (Magnification ×130; reduced 2/7.) *D*, Two hypertrophied pancreatic arterioles showing healing with resolution of inflammatory exudate and proliferation of connective tissue. (Magnification ×550; reduced 2/7.)

normal in rats with pedicle ligation, whereas in the enucleated series it was irregular, with formation of small cortical adenoma and of a central core of fibrous tissue, calcium salts, and altered blood.

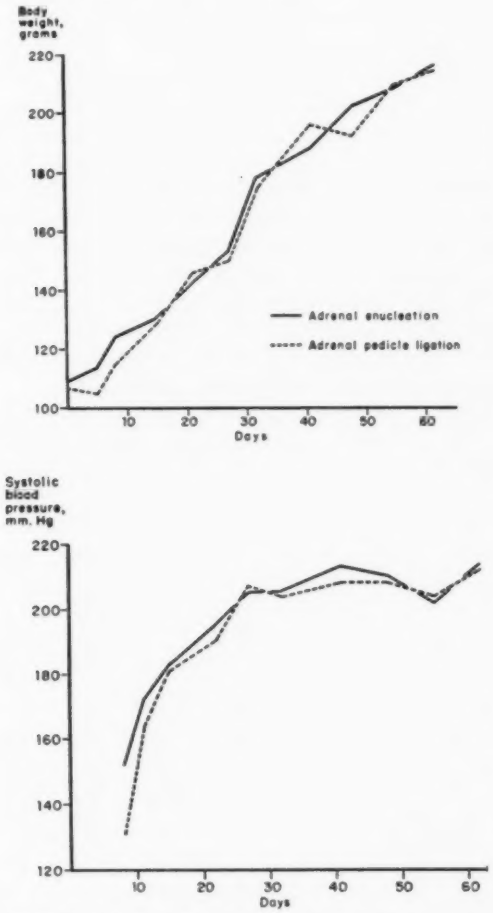

Fig. 2.—Comparative body weight and blood pressure changes in rats with adrenal enucleation and in rats with ligated adrenal pedicle.

Second Experimental Series.—In this series, mortality following pedicle ligation was less than in the first series; only 2 animals out of 12 died during the first postoperative week. The survivors showed initial weight loss but soon began to grow at the rate similar to that in animals with adrenal enucleation (Fig. 2). Hence, it appears that the technique of ligation used in this group caused only a little more adrenal insufficiency than resulted from enucleation. In the 10 survivors, the blood pressure rose sharply to hypertensive

levels at about the fifteenth day (Fig. 2); on the thirtieth day all the rats but one were consistently hypertensive and 3 had pressures of more than 200 mm. Hg. This sequence of events was similar to that in rats with adrenal enucleation; all these survived the operative procedures, and on the thirtieth day all had pressures higher than 150 mm. Hg, 4 of them with pressures of more than 200 mm. Hg.

On the sixtieth postoperative day, surviving animals of both groups were evenly divided in 2 new groups of 8 animals each, composed respectively of 4 rats with enucleated and 4 with ligated adrenals. The saline given as drink was replaced either with tap water or aqueous hydralazine. At the next determination of arterial pressure, it was found to have decreased substantially in both groups regardless of the operative procedure, and only to a slightly greater degree in those given hydralazine (mean decrease of 20 and 37 mm. Hg, respectively in the tap water group and the hydralazine group). On the ninetieth day 2 rats of the group receiving tap water and 5 of the groups receiving hydralazine were normotensive (systolic pressure less than 150 mm. Hg), and none of either group had pressures greater than 180 mm. Hg. At autopsy, heart and kidney weights were equal in both groups.

Microscopically, the striking changes caused by treatment with water alone or with added hydralazine consisted in the absence of progressive mesenteric arteritis and in evidences of healing of these lesions, with replacement of the inflammatory exudate with collagenous and elastic fibers (Fig. 1). Nephrosclerosis persisted in the kidneys. In the absence of a 90-day untreated control group, it was impossible to evaluate the effect of treatment on the renal vascular bed.

DISCUSSION

The experiments show a striking parallelism between the effects of adrenal pedicle ligation and those of adrenal enucleation with regard to the occurrence of hypertension and hypertensive vascular disease. This similarity indicates that the hypertension resulting from these procedures depends on a similar mechanism. The morphology of the adrenals regenerating after pedicle ligation is, except for the absence of medullary cells, normal. This in turn demonstrates that the enucleation hypertension is not dependent upon adenomatous regeneration, with its possible implication of abnormal steroid synthesis in these areas. Indeed, as demonstrated elsewhere,[7] the secretion patterns of enucleated regenerating adrenals are essentially normal in distribution although somewhat deficient in quantitative response to ACTH stimulation.

The experience confirms the findings of Poumeau-Delille and co-workers,[3] although this was only an incidental finding in his series of rats with adrenal pedicle ligation. The greater frequency of hypertension in the present experiments is presumably dependent in part upon the associated uninephrectomy and also, on the use of young animals, which, as noted above, are more susceptible to enucleation hypertension.

The means of blood pressures in control sham-operated rats and in enucleated rats given tap water were nearly the same in Skelton's series; however,

in 1 of 10 enucleated tap water treated animals, he did note slight nephrosclerosis, suggesting that this animal might have been hypertensive.[8] Our experience confirms the view that enucleation, and also ligation hypertension, are greatly enhanced upon provision for excess NaCl. However, this is not unequivocally an absolute dependence since mild hypertension and hypertensive vascular disease may appear in ligated or enucleated animals given no more salt than that in the daily ration of Purina Fox Chow. Salt enhancement is seen in establishing the onset of hypertension; it participates to a great degree also in the maintenance of hypertension since substitution of tap water for saline is only a little less effective in causing remission of hypertension than this plus the addition of hydralazine, a potent antipressor agent.

The vascular lesions occurring during the hypertensive disease consequent on adrenal pedicle ligation are identical with those seen after adrenal enucleation and require no further comment or description.[9] The remission of the mesenteric arteritis observed in hypertensive animals treated with tap water or tap water plus hydralazine is significant in that it further bears out the association between hypertension and hypertensive disease and also confirms our view that the mesenteric lesions of the rat are expressions of hypertensive vascular disease and hence not comparable with periarteritis nodosa.

The pathogenesis of the hypertension elicited by adrenal enucleation or pedicle ligation remains obscure. The experiments with pedicle ligation are consistent with our previously stated concept[2] that the hypertension consequent on enucleation may represent a state of vascular hyperactivity to the returning normal steroid secretions, this hyperactivity having developed during the period of postoperative adrenal insufficiency. They further show that the period of insufficiency is not a critical one, since insufficiency persists longer after ligation than after enucleation. However, as indicated previously, the evidence to support this concept is only indirect.

SUMMARY

1. Ligation of the adrenal vascular pedicle, like adrenal enucleation, induces an hypertensive state in uniadrenalectomized uninephrectomized rats.

2. The hypertension consequent on both procedures is greatly enhanced by provision of excess sodium chloride, but it is not an absolute dependence.

3. The remission of the hypertension in either group elicited by substitution of water or water plus hydralazine for saline is associated with remission and repair of mesenteric arteritis.

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REFERENCES

1. Skelton, F. R.: Development of Hypertension and Cardiovascular-Renal Lesions During Adrenal Regeneration, Proc. Soc. Exper. Biol. & Med. 90: 342-346, 1955.
2. Masson, G. M. C., and Corcoran, A. C.: Hormonal Influences on Adrenal Regeneration, Arch. Int. Pharmacodyn. In press.
3. Poumeau-Delille, G., Fells, E., and Kijewska, M.: Ligadura del pediculo vascular di la suprarrenal en la rata blanca, Rev. Soc. Argent. Biol. 30: 123-126, 1954.

4. Skelton, F. R., and Guillebeau, F.: The Influence of Age on Development of Adrenal-Regeneration Hypertension, Endocrinology **59**: 201-212, 1956.
5. Masson, G. M. C., del Greco, F., Olmsted, F., and Corcoran, A. C.: Graphic Measurements of Arterial Pressure in the Unanesthetized Rat, J. LAB. & CLIN. MED. **41**: 729-737, 1953.
6. Masson, G. M. C., Hazard, J. B., Corcoran, A. C., and Page, I. H.: Experimental Vascular Disease Due to Desoxycorticosterone and Anterior Pituitary Factors, A. M. A. Arch. Path. **49**: 641-664, 1950.
7. Masson, G. M. C., Koritz, S. B., and Peron, F. G.: Corticosteroid Formation in Regenerating Rat Adrenals, Endocrinology **62**: 229-233, 1958.
8. Skelton, F. R.: Adrenal-Regeneration Hypertension and Factors Influencing Its Development, A. M. A. Arch. Int. Med. **98**: 449-462, 1956.
9. Skelton, F. R.: Experimental Hypertensive Vascular Disease Accompanying Adrenal Regeneration in the Rat, Am. J. Path. **32**: 1037-1053, 1956.

LABORATORY METHODS

A HISTOPLASMIN-LATEX AGGLUTINATION TEST
I. Results With Animal Sera

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THE antibody response to infections with *Histoplasma capsulatum* has been studied most extensively with 3 types of tests: collodion agglutination,[1-6] precipitin,[7] and complement fixation.[8-12] All of these procedures have presented difficulties to the small laboratory with limited personnel and facilities. The collodion agglutination test, in our experience, has been very useful in the detection of early active histoplasmosis, and is employed routinely in this laboratory concurrently with the yeast-phase complement fixation test.[8-10] Although the collodion test is comparatively simple to perform, the preparation of the collodion particles has deterred some laboratories from undertaking this procedure. Recent studies[13, 14] with commercially produced polystyrene latex particles in the latex-fixation test for rheumatoid arthritis suggested their use as a "carrier" for the histoplasmin antigen in a manner analogous to the collodion particle test. The purpose of this report is to compare an agglutination test using histoplasmin-sensitized latex particles to the collodion agglutination test for histoplasmosis.

MATERIALS AND METHODS

Latex Particles.—Polystyrene latex particles* were in the form of a thick, white semifluid suspension when received. The suspension contained 27.6 per cent total solids, and was composed of uniformly distributed, discrete particles about one micron in diameter. A stock suspension was prepared by diluting one volume of this material with approximately 16 volumes of distilled water. The exact amount of distilled water used was such that a subsequent 1:100 dilution of the stock suspension yielded a final turbidity of 12 per cent light transmission in a Leitz Ruoy-photrometer at 535 mμ. This opacity corresponds approximately to the No. 10 MacFarland nephelometer tube. The stock latex suspension (1:17 dilution) was stored at 2° to 4° C.

Histoplasmin.—Histoplasmin prepared in the usual manner[15] from *H. capsulatum*, strain G-13, was used in the majority of the studies. Commercial skin test histoplasmin†

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was used in a sufficient portion of the work to ensure that this type of material could be used satisfactorily.

The Histoplasmin-Latex Test.—Antigen was prepared 1 to 2 hours before use by adding 0.5 ml. of the optimal dilution of histoplasmin and 0.1 ml. of stock latex suspension to 9.4 ml. of physiologic saline. A control antigen was prepared at the same time by mixing 9.9 ml. of saline and 0.1 ml. of stock latex suspension. Both suspensions were washed immediately prior to use by centrifuging at 2,400 r.p.m. for 12 minutes and resuspending in saline to the original volume. Serial twofold dilutions of serum inactivated at 56° C. for 30 minutes were prepared in saline in a final volume of 0.5 ml. A sufficient amount of the lowest dilution was prepared to provide an equal quantity for a control tube. Sensitized latex particles were added to all the tubes except the control tube which received unsensitized particles. All tubes were mixed thoroughly and kept at room temperature for 2 hours and then centrifuged at 2,000 r.p.m. for 5 minutes in an International horizontal centrifuge (Size 2). Readings were then made with the aid of a microscope mirror by gentle flicking or tapping of the tubes. Agglutinations were considered to be 4+ when most of the agglutinated particles were 1 to 2 mm. in size. Weaker reactions were graded as 3+, 2+, or 1+ depending on the size of the clumps and the appearance of the background as in any agglutination test. In the 1+ reaction, the agglutination was fine, but readily distinguishable from the control tube. Doubtful tubes which required more lengthy comparison with the control were read as ±.

Certain of the above conditions for performing the test were found to be not critical, and could be varied without appreciably affecting results. For example, the concentration of latex particles in the antigen could be varied as much as 25 per cent more or less than the recommended concentration. It was found further that antigen could be used any time from 1 to 3 hours after mixing. Inactivation of serum samples was found to be not necessary although control tubes in tests carried out on inactivated samples were somewhat smoother. Fresh physiologic saline proved to be suitable for use as a diluent in the test. It was not found necessary to use the borate buffer recommended in the latex-fixation test for rheumatoid arthritis.[13, 14]

The above optimal conditions for performing the histoplasmin-latex test were established by the use of serum specimens from artificially infected monkeys, immunized rabbits, and from known human cases. The same technique proved to be applicable to sera from all 3 species.

The Collodion Agglutination Test.—Procedures used in the preparation of materials and in the performance of collodion agglutination tests have been described previously.[1-4]

RESULTS

Standardization of Antigen.—The optimal dilution of histoplasmin was determined by titrating a known positive serum sample against latex particles sensitized with varying dilutions of histoplasmin, as shown in Table I. A total of 16 such cross titrations were carried out with strongly and weakly positive monkey, rabbit, and human sera. It was found that the same amount of histoplasmin was suitable for detecting antibody in both strongly and weakly positive sera. It was found further that there were no species differences in that the optimal dilution of histoplasmin was the same regardless of the species source of the serum. The optimal dilution of the current lot of G-13 histoplasmin prepared in this laboratory was arbitrarily selected as 1:300 although little difference was noted within a range of 1:200 to 1:600 (Table I); 1:50 was the optimal dilution of the commercial skin test histoplasmin (Lilly).

TABLE I. DETERMINATION OF OPTIMAL DILUTION OF HISTOPLASMIN (G-13) FOR LATEX AGGLUTINATION USING POSITIVE MONKEY SERUM

DILUTION OF HISTOPLASMIN BEFORE MIXTURE WITH 9.5 ML. LATEX SUSPENSION* (0.5 ML.)	FINAL HISTOPLASMIN DILUTION (IN 10 ML.)	SERUM DILUTIONS (0.5 ML.)								
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
1:2.5	1:50	4+	4+	4+	4+	3+	2+	1+	–	–
1:5	1:100	4+	4+	4+	4+	4+	3+	2+	–	–
1:10	1:200	4+	4+	4+	4+	4+	3+	3+	1+	–
1:15	1:300†	4+	4+	4+	4+	4+	3+	3+	1+	±
1:20	1:400	4+	4+	4+	4+	4+	3+	2+	1+	–
1:30	1:600	4+	4+	4+	4+	4+	3+	2+	1+	–
1:40	1:800	4+	4+	4+	4+	4+	2+	1+	±	–
1:80	1:1,600	4+	4+	3+	3+	3+	1+	–	–	–
1:160	1:3,200	4+	4+	3+	3+	2+	±	–	–	–
None‡	None	–	–	–	–	–	–	–	–	–

*Nine and four-tenths milliliters saline and 0.1 ml. stock latex suspension.
†Optimal dilution of histoplasmin selected as 1:300.
‡Latex control—particles without histoplasmin.

TABLE II. COMPARATIVE RESULTS IN THE HISTOPLASMIN-LATEX AGGLUTINATION TEST USING WASHED AND UNWASHED ANTIGEN

SERA		ANTIGEN NOT WASHED — RECIPROCAL OF TITER									ANTIGEN WASHED — RECIPROCAL OF TITER									
		5	10	20	40	80	160	320	640	C*	5	10	20	40	80	160	320	640	1,280	C*
Positive (Infected (Monkey)	1	4+	3+	2+	2+	1+	±	–	–	–	4+	4+	4+	3+	2+	1+	±	–	–	–
	2	4+	4+	3+	2+	1+	1+	–	–	–	4+	4+	3+	2+	2+	1+	±	–	–	–
	3	4+	4+	4+	4+	3+	2+	1+	–	–	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
	4	4+	4+	4+	3+	2+	1+	±	–	–	4+	4+	4+	3+	3+	3+	2+	±	–	–
Negative	5	2+	1+	±	–	–	–	–	–	–	2+	2+	1+	–	–	–	–	–	–	–
(Monkey)	6	1+	±	–	–	–	–	–	–	–	1+	1+	–	–	–	–	–	–	–	–
Positive (Immunized Rabbit)	1	4+	4+	4+	4+	3+	2+	1+	1+	–	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
	2	4+	4+	4+	4+	3+	3+	2+	1+	–	4+	4+	4+	4+	4+	3+	2+	2+	1+	–
	3	4+	4+	4+	4+	3+	2+	1+	±	–	4+	4+	4+	4+	3+	3+	1+	±	–	–
	4	4+	4+	4+	3+	3+	1+	–	–	–	4+	4+	4+	4+	3+	1+	±	–	–	–
	5	4+	4+	3+	2+	1+	–	–	–	–	4+	4+	4+	3+	2+	1+	–	–	–	–
	6	4+	3+	3+	1+	±	–	–	–	–	4+	4+	3+	2+	1+	–	–	–	–	–
	7	3+	2+	1+	±	–	–	–	–	–	4+	3+	3+	2+	1+	–	–	–	–	–
	8	2+	1+	1+	–	–	–	–	–	–	3+	3+	2+	1+	–	–	–	–	–	–
Negative	9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(Rabbit	10	1+	±	–	–	–	–	–	–	–	1+	1+	±	–	–	–	–	–	–	–
Sera)	11	1+	1+	±	–	–	–	–	–	–	1+	1+	±	–	–	–	–	–	–	–

*Latex control—particles without histoplasmin.

Washing of Antigen.—It was found that the sensitivity of the latex agglutination test was increased slightly by washing the antigen once in physiologic saline immediately prior to use in the test. Washing was accomplished by centrifuging at 2400 r.p.m. for 12 minutes in 18 by 150 mm. Pyrex tubes and resuspending in saline to the original volume, 10 ml. The sedimented material was distributed evenly by 8 to 10 mixings with a 10 ml. pipette. Table II presents the results of parallel tests carried out on positive and negative rabbit and monkey sera using washed and unwashed antigens.

Temperature and Duration of Incubation.—The optimal incubation temperature and time for histoplasmin-latex agglutination tests was established

by carrying out 15 separate titrations of the same positive serum sample and incubating simultaneously in water baths at different temperatures for different lengths of time. Five of the 15 tests were incubated at 25° C., 5 at 37° C., and 5 at 56° C. One test from each group was centrifuged and read after 30 minutes and 1, 2, 3, and 4 hours' incubation. The results, as depicted in Table III, are representative of this study. It can be seen that incubation at either room temperature or 37° C. for 2 to 4 hours was suitable. For convenience, the same 2-hour, room-temperature incubation period which is used in the collodion agglutination test was adopted as the standard procedure for the latex agglutination test.

TABLE III. COMPARISON OF DIFFERENT INCUBATION TIMES AND TEMPERATURES IN HISTOPLASMIN-LATEX AGGLUTINATION TEST USING POSITIVE RABBIT SERUM

INCUBATION		RECIPROCAL OF TITER									
TEMPERATURE (°C.)	TIME (HR.)	10	20	40	80	160	320	640	1,280	2,560	C*
25	0.5	4+	4+	4+	4+	3+	2+	1+	±	–	–
	1	4+	4+	4+	4+	4+	3+	2+	1+	–	–
	2	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	3	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	4	4+	4+	4+	4+	4+	3+	1+	1+	–	–
37	0.5	4+	4+	4+	4+	3+	2+	1+	–	–	–
	1	4+	4+	4+	4+	3+	2+	1+	1+	–	–
	2	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	3	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	4	4+	4+	4+	4+	4+	2+	1+	±	–	–
56	0.5	4+	3+	3+	2+	1+	1+	–	-	–	–
	1	4+	3+	3+	2+	2+	1+	–	–	–	–
	2	4+	4+	4+	2+	1+	1+	–	–	–	–
	3	4+	4+	3+	2+	1+	±	–	–	–	–
	4	4+	4+	3+	2+	1+	–	–	–	–	–

*Latex control—particles without histoplasmin and 1:5 serum dilution.

Centrifugation Prior to Reading of Tests.—Preliminary observation indicated that centrifugation of histoplasmin-latex agglutination tests was necessary in order to obtain clear-cut, readable reactions. The speed and duration of the centrifugation was found to be critical in that excessive speeds and times resulted in nonspecific agglutination in control tubes. The optimal conditions were established by centrifuging aliquot samples at different speeds for varying lengths of time in an International, Size 2, horizontal centrifuge (Table IV). As a result of this study, centrifugation at 2,000 r.p.m. for 5 minutes was selected for use in histoplasmin-latex tests.

Repeatability of Histoplasmin-Latex Agglutination Tests.—The reproducibility of results of any serologic technique is an important criterion of the value of that procedure. Table V lists the results obtained with 6 representative positive samples in tests performed on different days. Although minor differences can be noted, the degree of variation between repeated tests is not out of the range of probability.

Results With Animal Sera.—Histoplasmin-latex and histoplasmin-collodion agglutination tests were carried out on a total of 81 serum specimens from

TABLE IV. EFFECT OF SPEED AND DURATION OF CENTRIFUGATION ON HISTOPLASMIN-LATEX AGGLUTINATION TESTS USING POSITIVE RABBIT SERUM

CENTRIFUGATION		RECIPROCAL OF TITER									
SPEED (R.P.M.)	TIME (MIN.)	10	20	40	80	160	320	640	1,280	2,560	C*
1,500	1	3+	3+	2+	2+	1+	1+	–	–	–	–
1,500	3	4+	3+	2+	2+	2+	1+	±	–	–	–
1,500	5	4+	4+	4+	3+	2+	1+	1+	±	–	–
1,500	7	4+	4+	4+	4+	3+	2+	1+	1+	–	–
1,500	9	4+	4+	4+	4+	4+	2+	2+	1+	–	–
1,500	11	4+	4+	4+	4+	4+	2+	1+	±	±	±
2,000	1	3+	3+	3+	2+	1+	1+	–	–	–	–
2,000	3	4+	4+	3+	3+	2+	1+	1+	–	–	–
2,000	5†	4+	4+	4+	4+	4+	3+	2+	1+	–	–
2,000	7	4+	4+	4+	4+	4+	3+	2+	1+	±	±
2,000	9	4+	4+	4+	4+	4+	3+	3+	1+	±	±
2,000	11	4+	4+	4+	4+	4+	4+	3+	1+	1+	1+

*Latex control—particles without histoplasmin.
†Five minutes at 2,000 r.p.m. selected as optimal because of highest titer without nonspecific agglutination in control.

TABLE V. REPEATABILITY OF RESULTS OF HISTOPLASMIN-LATEX AGGLUTINATION TESTS USING POSITIVE MONKEY AND RABBIT SERA

SPECIMEN NUMBER	SAMPLE* NUMBER	RECIPROCAL OF TITER									
		5	10	20	40	80	160	320	640	1,280	C†
Monkey 1	1	4+	4+	4+	3+	2+	1+	±	–	–	–
	2	4+	4+	3+	2+	2+	1+	±	–	–	–
	3	4+	4+	4+	3+	2+	1+	–	–	–	–
	4	4+	4+	3+	3+	2+	1+	±	–	–	–
Monkey H	1	4+	4+	4+	4+	4+	4+	3+	1+	–	–
	2	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	3	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
	4	4+	4+	4+	4+	4+	3+	2+	1+	–	–
Rabbit 1	1	4+	4+	4+	4+	4+	3+	2+	2+	1+	–
	2	4+	4+	4+	4+	4+	3+	2+	1+	1+	–
	3	4+	4+	4+	4+	4+	3+	3+	2+	1+	–
	4	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	5	4+	4+	4+	4+	3+	3+	2+	1+	1+	–
Rabbit 2	1	4+	4+	4+	4+	4+	3+	2+	2+	1+	–
	2	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
	3	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	4	4+	4+	4+	4+	4+	3+	3+	2+	1+	–
	5	4+	4+	4+	4+	4+	3+	1+	1+	±	–
	6	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
Rabbit HF	1	4+	4+	4+	4+	4+	3+	1+	–	–	–
	2	4+	4+	4+	4+	3+	2+	1+	1+	–	–
	3	4+	4+	4+	4+	4+	3+	2+	1+	–	–
Rabbit 6	1	4+	3+	3+	2+	1+	–	–	–	–	–
	2	4+	4+	4+	2+	1+	–	–	–	–	–
	3	4+	4+	3+	2+	1+	±	–	–	–	–
	4	4+	4+	3+	3+	2+	1+	–	–	–	–

*Every number represents a sample of the respective serum specimen.
†Latex control—particles without histoplasmin.

normal and artificially infected monkeys and dogs and from normal and immunized rabbits. These sera, taken in connection with other studies,[16, 17] varied from serologically negative to strongly positive. The monkey and dog sera were from animals bled before and at intervals after intratracheal or intranasal administration of *H. capsulatum* in the yeast phase. The rabbit sera were from normal animals and from animals immunized by a series of intravenous inoculations of killed yeast-phase organisms.[8]

TABLE VI. COMPARISON OF RESULTS OBTAINED WITH POSITIVE AND NEGATIVE MONKEY SERA BY THE HISTOPLASMIN-LATEX AND THE HISTOPLASMIN-COLLODION AGGLUTINATION TESTS

	SPECIMEN NUMBER	LATEX AGGLUTINATION — RECIPROCAL OF TITER										COLLODION AGGLUTINATION — RECIPROCAL OF TITER									
		5	10	20	40	80	160	320	640	1,280	C*	5	10	20	40	80	160	320	640	1,280	C†
Positive Sera	1	4+	4+	4+	4+	4+	4+	3+	2+	1+	–	4+	4+	4+	4+	4+	4+	3+	1+	–	–
	2	4+	4+	4+	4+	4+	4+	3+	2+	1+	–	4+	4+	4+	4+	4+	3+	3+	1+	±	–
	3	4+	4+	4+	3+	3+	3+	2+	±	–	–	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	4	4+	4+	4+	3+	3+	1+	1+	–	–	–	4+	4+	4+	4+	4+	3+	2+	1+	–	–
	5	3+	3+	2+	2+	1+	–	–	–	–	–	3+	3+	3+	2+	1+	±	–	–	–	–
	6	4+	3+	3+	2+	1+	–	–	–	–	–	4+	4+	3+	2+	1+	±	–	–	–	–
	7	4+	4+	4+	2+	2+	1+	–	–	–	–	4+	3+	3+	2+	2+	1+	–	–	–	–
	8	4+	4+	4+	3+	2+	1+	±	–	–	–	4+	4+	4+	3+	2+	1+	–	–	–	–
	9	4+	4+	4+	4+	3+	2+	1+	–	–	–	4+	4+	4+	3+	2+	1+	–	–	–	–
	10	4+	4+	4+	4+	3+	2+	1+	–	–	–	4+	4+	3+	2+	1+	±	–	–	–	–
	11	4+	4+	3+	2+	1+	–	–	–	–	–	4+	3+	3+	2+	1+	–	–	–	–	–
Negative Sera	12	2+	2+	1+	–	–	–	–	–	–	–	2+	1+	1+	±	–	–	–	–	–	–
	13	1+	1+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	14	1+	±	–	–	–	–	–	–	–	–	1+	±	–	–	–	–	–	–	–	–
	15	1+	1+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	16	1+	1+	±	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	17-27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*Latex control—particles without histoplasmin.
†Collodion control—particles without histoplasmin.

TABLE VII. COMPARISON OF RESULTS OBTAINED WITH POSITIVE AND NEGATIVE RABBIT SERA BY THE HISTOPLASMIN-LATEX AND THE HISTOPLASMIN-COLLODION AGGLUTINATION TESTS

	SPECIMEN NUMBER	LATEX AGGLUTINATION — RECIPROCAL OF TITER										COLLODION AGGLUTINATION — RECIPROCAL OF TITER									
		5	10	20	40	80	160	320	640	1,280	C*	5	10	20	40	80	160	320	640	1,280	C†
Positive Sera	1	4+	4+	4+	4+	4+	3+	2+	2+	1+	–	4+	4+	4+	4+	4+	3+	3+	2+	1+	–
	2	4+	4+	4+	4+	4+	3+	2+	2+	1+	–	4+	4+	4+	4+	4+	4+	3+	2+	1+	–
	3	4+	4+	4+	4+	3+	3+	1+	±	–	–	4+	4+	4+	4+	3+	3+	2+	±	–	–
	4	4+	4+	3+	3+	2+	1+	±	–	–	–	4+	4+	4+	3+	2+	1+	–	–	–	–
	5	4+	4+	4+	4+	3+	1+	±	–	–	–	4+	4+	4+	4+	3+	2+	1+	±	–	–
	6	4+	4+	3+	2+	1+	±	–	–	–	–	4+	4+	3+	2+	1+	–	–	–	–	–
	7	4+	4+	2+	1+	–	–	–	–	–	–	4+	4+	4+	3+	1+	–	–	–	–	–
	8	4+	4+	4+	3+	3+	2+	1+	–	–	–	4+	4+	4+	4+	4+	3+	3+	2+	1+	–
	9	4+	4+	4+	4+	4+	3+	3+	2+	1+	–	4+	4+	4+	4+	4+	3+	3+	2+	1+	–
	10	4+	4+	4+	4+	3+	3+	2+	1+	–	–	4+	4+	4+	4+	4+	3+	2+	1+	–	–
	11	4+	4+	3+	3+	2+	1+	–	–	–	–	4+	4+	4+	3+	3+	1+	±	–	–	–
	12	4+	3+	3+	3+	2+	1+	–	–	–	–	4+	3+	3+	2+	1+	–	–	–	–	–
	13	4+	4+	4+	4+	4+	3+	2+	1+	–	–	4+	4+	4+	4+	4+	3+	2+	1+	±	–
	14	4+	4+	4+	4+	4+	3+	1+	–	–	–	4+	4+	4+	4+	4+	3+	3+	1+	–	–
Negative Sera	15	1+	±	–	–	–	–	–	–	–	–	2+	1+	1+	±	–	–	–	–	–	–
	16	1+	1+	–	–	–	–	–	–	–	–	2+	2+	1+	±	–	–	–	–	–	–
	17	1+	±	–	–	–	–	–	–	–	–	1+	1+	–	–	–	–	–	–	–	–
	18	2+	1+	±	–	–	–	–	–	–	–	2+	1+	1+	–	–	–	–	–	–	–
	19	±	±	–	–	–	–	–	–	–	–	1+	1+	±	–	–	–	–	–	–	–
	20	2+	1+	1+	–	–	–	–	–	–	–	3+	2+	1+	–	–	–	–	–	–	–
	21	2+	1+	1+	–	–	–	–	–	–	–	3+	2+	1+	–	–	–	–	–	–	–
	22	2+	1+	±	–	–	–	–	–	–	–	2+	1+	1+	–	–	–	–	–	–	–
	23-35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*Latex control—particles without histoplasmin.
†Collodion control—particles without histoplasmin.

As can be seen from Tables VI, VII, and VIII, agreement between the two tests was good. The histoplasmin-latex test proved to be as sensitive and as specific as the collodion agglutination test in detecting antibodies in positive animal sera. Two possible exceptions were dog serum specimens 3 and 6 which have repeatedly failed to give 4+ reactions in the latex test (Table VIII). Weak reactions were observed in both tests with serum samples from normal animals of all 3 species. All of the 13 normal dog serum samples agglutinated sensitized latex and collodion particles in low dilution. Reactivity of normal serum was observed less frequently with samples from rabbits and monkeys, especially the latter.

TABLE VIII. COMPARISON OF RESULTS OBTAINED WITH POSITIVE AND NEGATIVE DOG SERA BY THE HISTOPLASMIN-LATEX AND THE HISTOPLASMIN-COLLODION AGGLUTINATION TESTS

		LATEX AGGLUTINATION								COLLODION AGGLUTINATION							
	SPECIMEN NUMBER	RECIPROCAL OF TITER								RECIPROCAL OF TITER							
		5	10	20	40	80	160	320	C*	5	10	20	40	80	160	320	C†
Positive Sera	1	4+	4+	4+	4+	3+	1+	1+	–	4+	4+	4+	4+	3+	2+	1+	–
	2	4+	4+	3+	2+	1+	±	–	–	4+	4+	4+	2+	1+	±	–	–
	3	3+	3+	3+	2+	1+	1+	–	–	4+	4+	4+	3+	2+	1+	–	–
	4	4+	4+	3+	3+	2+	1+	–	–	4+	4+	4+	4+	3+	2+	1+	–
	5	4+	4+	3+	2+	1+	±	–	–	4+	4+	4+	3+	1+	1+	–	–
	6	3+	3+	3+	3+	2+	1+	–	–	4+	4+	4+	4+	3+	2+	1+	–
Negative Sera	7	3+	2+	1+	±	–	–	–	–	3+	2+	1+	1+	–	–	–	–
	8	2+	1+	1+	–	–	–	–	–	3+	2+	±	–	–	–	–	–
	9	1+	1+	±	–	–	–	–	–	2+	2+	1+	1+	–	–	–	–
	10	2+	2+	1+	–	–	–	–	–	3+	2+	1+	±	–	–	–	–
	11	1+	1+	±	–	–	–	–	–	2+	1+	1+	–	–	–	–	–
	12	2+	1+	±	–	–	–	–	–	1+	1+	–	–	–	–	–	–
	13	2+	1+	1+	–	–	–	–	–	3+	2+	1+	1+	–	–	–	–
	14	3+	2+	1+	–	–	–	–	–	3+	3+	2+	1+	–	–	–	–
	15	1+	±	–	–	–	–	–	–	3+	2+	1+	–	–	–	–	–
	16	2+	1+	1+	–	–	–	–	–	2+	2+	1+	–	–	–	–	–
	17	2+	1+	1+	–	–	–	–	–	2+	2+	1+	–	–	–	–	–
	18	3+	2+	1+	±	–	–	–	–	2+	2+	2+	1+	–	–	–	–
	19	1+	–	–	–	–	–	–	–	3+	1+	1+	–	–	–	–	–

*Latex control—particles without histoplasmin.
†Collodion control—particles without histoplasmin.

DISCUSSION

The results described herein suggest that polystyrene latex particles can be employed successfully in an agglutination test with histoplasmin antigen. Comparison with the collodion agglutination test reveals marked parallelism. Considerable flexibility is allowable in reference to optimal dilution of histoplasmin and latex, and in time and temperature of incubation. These considerations plus the availability of latex particles and histoplasmin simplify the problems of a smaller laboratory desiring to perform this test in the serologic diagnosis of histoplasmosis. Preliminary results with human sera indicate that this test is applicable in the diagnosis of acute histoplasmosis. These data will be discussed in a subsequent report.[18]

SUMMARY

A new serologic technique for the detection of antibodies against *H. capsulatum* is presented. In the performance of this technique, polystyrene latex

particles are "coated" with histoplasmin and used in a simple tube agglutination test. The histoplasmin-latex agglutination test and the histoplasmin-collodion agglutination test showed comparable results from the standpoint of specificity and sensitivity in sera from infected dogs and monkeys and immunized rabbits.

The technical assistance of Joann Sparks and the suggestions of Dr. Melvin Rheins are gratefully acknowledged.

REFERENCES

1. Saslaw, S., and Campbell, C. C.: A Method for Demonstrating Antibodies in Rabbit Sera Against Histoplasmin by the Collodion Agglutination Technic, Proc. Soc. Exper. Biol. & Med. **68:** 559-562, 1948.
2. Saslaw, S., and Campbell, C. C.: A Collodion Agglutination Test for Histoplasmosis, Pub. Health Rep. **64:** 424-429, 1949.
3. Saslaw, S., and Campbell, C. C.: Serologic Studies in Histoplasmosis, Am. J. Pub. Health **40:** 427-435, 1950.
4. Saslaw, S., and Campbell, C. C.: Studies on the Stability of the Histoplasmin Collodion Agglutination Test, J. Lab. & Clin. Med. **35:** 780-785, 1950.
5. Saslaw, S.: The Collodion Agglutination Test, Proceedings of the Conference on Histoplasmosis, Public Health Monograph No. 39, pp. 123-128, 1952.
6. Saslaw, S., and Campbell, C. C.: Effect of Histoplasmin Skin Testing on Serologic Results, Proc. Soc. Exper. Biol. & Med. **82:** 689-691, 1953.
7. Salvin, S. B., and Furcolow, M. L.: Precipitins in Human Histoplasmosis, J. Lab. & Clin. Med. **43:** 259-274, 1954.
8. Saslaw, S., and Campbell, C. C.: The Use of Yeast Phase Antigens in a Complement Fixation Test for Histoplasmosis. I. Preliminary Results With Rabbit Sera, J. Lab. & Clin. Med. **33:** 811-818, 1948.
9. Campbell, C. C., and Saslaw, S.: The Use of Yeast Phase Antigens in a Complement Fixation Test for Histoplasmosis. II. Results with Ground Antigens, J. Lab. & Clin. Med. **33:** 1207-1211, 1948.
10. Campbell, C. C., and Saslaw, S.: The Use of Yeast Phase Antigens in a Complement Fixation Test for Histoplasmosis. III. Preliminary Results With Human Sera, Pub. Health Rep. **64:** 551-560, 1949.
11. Salvin, S. B.: Complement Fixation Studies in Experimental Histoplasmosis, Proc. Soc. Exper. Biol. & Med. **66:** 342-345, 1947.
12. Tenenberg, D. J., and Howell, A.: A Complement Fixation Test for Histoplasmosis. I. Technic and Preliminary Results in Animal Sera, Pub. Health Rep. **63:** 163-168, 1948.
13. Singer, J. M., and Plotz, C. M.: The Latex Fixation Test. I. Application to the Serologic Diagnosis of Rheumatoid Arthritis, Am. J. Med. **21:** 888-892, 1956.
14. Plotz, C. M., and Singer, J. M.: The Latex Fixation Test. II. Results in Rheumatoid Arthritis, Am. J. Med. **21:** 893-896, 1956.
15. Howell, A.: Studies of Fungus Antigens. I. Quantitative Studies of Cross Reactions Between Histoplasmin and Blastomycin in Guinea Pigs, Pub. Health Rep. **62:** 631-651, 1947.
16. Saslaw, S., Maurice, G. E., and Cole, C. R.: Experimental Histoplasmosis in Large Animals, J. Lab. & Clin. Med. **46:** 948, 1955.
17. Saslaw, S., and Cole, C. R.: Unpublished observations.
18. Carlisle, H. N., and Saslaw, S.: A Histoplasmin-Latex Agglutination Test. II. Results With Human Sera. (To be published.)

USE OF PHAGE-IMPREGNATED PAPER DISKS FOR BACTERIAL SPECIFICITY TESTS

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PAPER disk methods to determine susceptibility of organisms to antibiotics have proved to be rapid, simple, and practical. Such methods have been successfully adapted in our laboratory to differentiate bacterial strains by the use of phage.

In the usual method of characterizing a bacterial strain with respect to susceptibility and resistance to specific phage types, it is necessary that the added phage suspension be limited to a small droplet to avoid excessive "running" of the liquid over the agar surface. Also, droplets must be placed several centimeters apart to avoid overlapping of lysed areas, and care must be taken to label the point at which each specific phage suspension is added. The disk method described eliminates the need for liquid test suspensions and simplifies phage typing to the level of antibiotic sensitivity testing.

MATERIALS AND METHODS

Phages and Host Systems.—Phages prepared and tested were the seven T phages of *Escherichia coli* B, PLT22 phage of *Salmonella typhimurium* LT2, Vi phage of *Salmonella typhosa* E, six XP phages of *Xanthomonas pruni*, gamma phage against 11 strains of *Bacillus anthracis*, and 16 staphylococcal phages against 35 strains of *Micrococcus pyogenes.**

Disk Preparation.—Two different disk systems were used. In one case, disks of 7 mm. diameter were punched out of desk blotters. A different color was used for each phage type, thus eliminating the necessity for numbering the position of the phages on the Petri plate (see Fig. 1). Individual disks were saturated with about 0.3 ml. of phage suspension and dried at 37° C.

In the second case, Multidisks† containing 9 tips were used (see Fig. 2). It was necessary to increase the viscosity of the phage suspension to avoid migration of the phage along the edge of the filter paper when using the multidisks. The addition of albumin to a final concentration of 15 per cent was found to be superior to other thickening and drying substances tested. A microdrop of phage suspension was applied to each tip of the multidisk with a capillary tube and allowed to dry at 37° C.

Procedure.—Petri plates were prepared using the double-layer method with the second layer inoculated with host cells. Three plates were prepared with each different host.

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*The staphylococcal phages were obtained from the Laboratory Division, Hospital for Joint Diseases, New York, N. Y.

†Multidisks were obtained from Consolidated Laboratories, Inc., Chicago Heights, Ill.

Individual disks were placed on one plate, a Multidisk on the second plate, and microdrops of the phage suspension on the third plate. A maximum of 9 individual disks and 9 microdrops of phage suspension was used per plate. The forceps used for placing the disks on the plates were flamed after each use to avoid unwanted transfer or contamination of phage. The plates spotted with the microdrops were used as controls. The plates were incubated at 37° C. for 24 hours.

In all experiments, control plates were prepared in a manner identical to the above, except that for each case where a disk was used, a droplet of phage was substituted in the control.

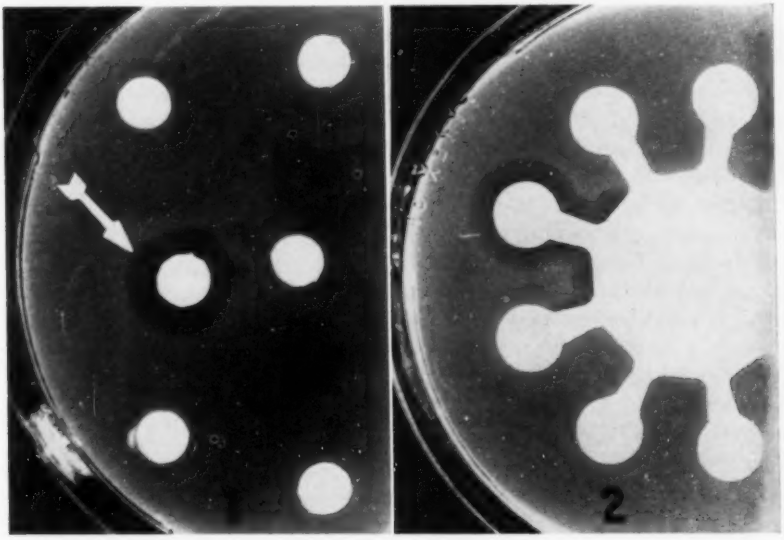

Fig. 1. Fig. 2.

Fig. 1.—Phage-impregnated blotter paper disks. Clear zone surrounding disk indicates lysis of bacteria that have been seeded in the agar layer of the Petri plate. Each disk is a different color, but this is not visible because of the shadowgraphing method used.[3]

Fig. 2.—Each tip of the multidisk is impregnated with a different phage type. The arrows point to the disk or tip containing the phage specific for the bacterial strain seeded in the agar.

RESULTS AND DISCUSSION

Results were recorded by comparing samples in which the disk method was used with the conventional method based upon the use of microdrops of phage to lyse bacterial cells.

Both types of impregnated disks, as well as liquid samples of phage, showed good lytic properties after storage for 90 days at 4° C., –10° C., and room temperature varying between 23° and 42° C. Thus, under the conditions of storage, the disks proved to be equally as stable as liquid samples of phage.

In order to evaluate the sensitivity of the disk method, dilutions of phage were placed on disks to determine the minimum number of phage particles that would reveal a positive score. For the *Esch. coli* phage series, as few as 2×10^2 phages per disk still permitted a zone of lysis to appear; in all of the other cases,

including the staphylococcus system and the *B. anthracis* system, 4×10^3 phage particles per disk were necessary to reveal a clear zone of lysis. These values are higher than the lower limit that would be necessary to produce a clear zone by the microdrop technique (only a single particle is needed to produce a plaque). Phage stocks are usually prepared with titers of perhaps 10^6 particles per microdrop. Therefore, even though more particles may be needed on a disk than are needed in a droplet, this is not a handicap since the ability to place a large number of phage in a test sample is not a limiting or critical factor.

In phage typing of staphylococci, one of the distinguishing characteristics is the visual appearance of the lysis zone; some bacterial strains are lysed so that a confluent clear zone arises while other strains are lysed with a semiconfluent zone.[1] Both of these types of lysis in staphylococci were as readily visible when impregnated disks were used as in the case of microdrops. Also, in the case of the lytic pattern of gamma phage on *B. anthracis*, the clear zones were identical when the 2 methods were compared.[2]

From the above comparisons, it may be concluded that the use of impregnated paper disks for phage typing gives as satisfactory results as the use of microdroplets of phage suspensions. The disk technique, however, has an advantage inasmuch as a large number of disks may be prepared in advance, stored, and used when needed with a minimum of effort. Further, phage-impregnated disks have been used successfully for the storage of stock phage types and for shipment of phage stocks to other laboratories. As an example of the latter, impregnated disks were placed in sterile small envelopes and mailed in a regular envelope. After an ocean voyage the disks were used successfully for the initiation of fresh phage stocks and for phage typing.

SUMMARY

A method of using phage-impregnated paper disks for specificity tests of bacteria has been described. This method gave the same results as obtained by other investigators using staphylococcal and *B. anthracis* phages. The technique permitted preparation and storage of disks prior to testing. The same disks were used conveniently for storage of stocks and shipment of phages to other laboratories.

REFERENCES

1. Blair, J. E., and Carr, M.: The Bacteriophage Typing of Staphylococci, J. Infect. Dis. **93**: 1-13, 1953.
2. Brown, E. R., and Cherry, W. B.: Specific Identification of Bacillus anthracis by Means of a Variant Bacteriophage, J. Infect. Dis. **96**: 34-39, 1955.
3. Eisenstark, A., and Eisenstark, R.: Photography of Biological Materials Without a Camera, J. Biol. Photographic A. **21**: 6-11, 1953.

EXPERIENCE WITH THE TAMe ASSAY DURING ANTICOAGULANT THERAPY

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DESPITE the steadily increasing use of anticoagulant (prothrombin-depressant) drugs there still exist wide differences of opinion concerning their clinical usefulness and safety. Whereas some authorities in the fields of medicine and surgery believe them to be of little value, others consider them to be important therapeutic agents provided their administration is properly controlled. Just what constitutes proper control and how to achieve it most reliably and conveniently is still a matter of some debate. Contributing to the general confusion are a diversity of "prothrombin" assay procedures, a lack of uniformity in the type of reagents used, and, not least of all, a dearth of information concerning the specific and possibly different effects exerted by the various anticoagulants currently in use.

For the past 10 years we have controlled the administration of anticoagulant drugs to patients by the combined use of the one-stage and two-stage prothrombin assays.[1, 2] Although both these techniques were originally thought to provide specific, quantitative measurements of plasma prothrombin it has since become apparent that, under the limiting conditions found in therapy plasmas, results provided by these procedures are also influenced by factors other than prothrombin.[3-6] From our clinical experience obtained over the years, we have concluded that of the two methods the two-stage technique provides the more consistently reliable index for the control of therapy. The information provided by this test appears to be less subject to certain variations frequently associated with the one-stage assay, thus producing more satisfactory patient results.[7] Largely because of its greater technical complexity, however, the two-stage assay has not been generally accepted for routine clinical use.

The TAMe assay for thrombin as developed by Sherry and Troll[8] and subsequently adapted by Glueck, Sherry, and Troll[9] to measure plasma prothrombin is similar in principle to the two-stage technique in that it separates the two phases of the clotting mechanism (Fig. 1). However, instead of the thrombin formed during the first stage being added to fibrinogen, it is allowed to react with tosyl-arginine-methyl ester (TAMe). This results in the splitting

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of the tosyl-arginine ester linkage and the liberation of free tosyl-arginine which can then be determined by titration with standard alkali. Under suitable conditions the amount of tosyl-arginine formed is a function of the thrombin and, therefore, prothrombin concentration.

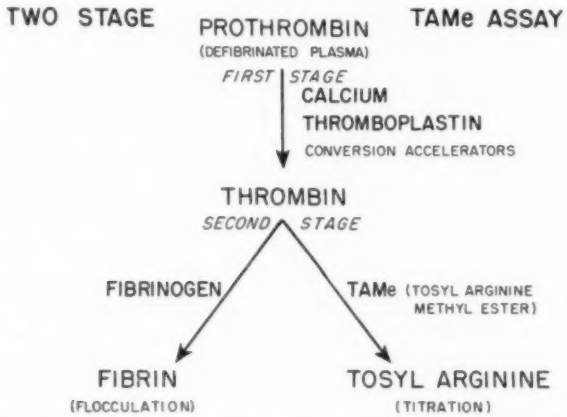

Fig. 1.—Schematic representation of two-stage and TAMe assays.

The TAMe assay was of particular interest to us since Glueck, Sherry, and Troll had presented preliminary evidence to indicate that with plasmas from patients treated with Dicumarol it produced results paralleling those obtained with the two-stage assay.[9] This finding, coupled with the sound chemical basis and relative simplicity of the TAMe method, suggested it as a promising alternative to the two-stage technique. It was decided, therefore, to compare the results obtained with the two assays when applied to plasmas from patients individually maintained on all presently used prothrombin-depressant drugs.

METHODS

Blood for this study was obtained from hospitalized patients of the Presbyterian-St. Luke's Hospital of Chicago who had been placed on 1 of 8 anticoagulant drugs. Blood was collected by the two-syringe technique, 9 parts of blood being added to 1 part of 3.2 per cent sodium citrate. The samples were centrifuged and the plasmas assayed within 4 hours of collection. The two-stage assay was carried out as described by Warner, Brinkhous, and Smith[10] and modified by Ware and Seegers[2] and the TAMe assay was done as outlined by Glueck, Sherry, and Troll.[9] The anticoagulant drugs used included Cyclocumarol, Dicumarol, Dipaxin, Marcumar, phenylindanedione, Tromexan, Sintrom, and Warfarin. Dosages varied according to drug and individual response of the patient. Factor V concentrates were prepared according to the method of Lewis and Ware.[11] Factor VII concentrates were obtained as described by Alexander, Goldstein, and Landwehr.[12] Beef lung thromboplastin was prepared according to Ware and Seegers,[2] beef brain thromboplastin as prepared according to De Nicola,[13] and human brain thromboplastin after Biggs and Macfarlane.[14]

RESULTS

The 2 assays were carried out on 546 plasmas from 60 patients maintained on anticoagulant therapy for varying periods of time. Additional determinations

were made on 155 plasmas obtained from a variety of patients for whom routine prothrombin determinations had been ordered. In our hands, a 100 per cent prothrombin level as determined by the two-stage method was equivalent to 50 TAMe units (s. e. ± 3.4 per cent) as measured by the TAMe assay.

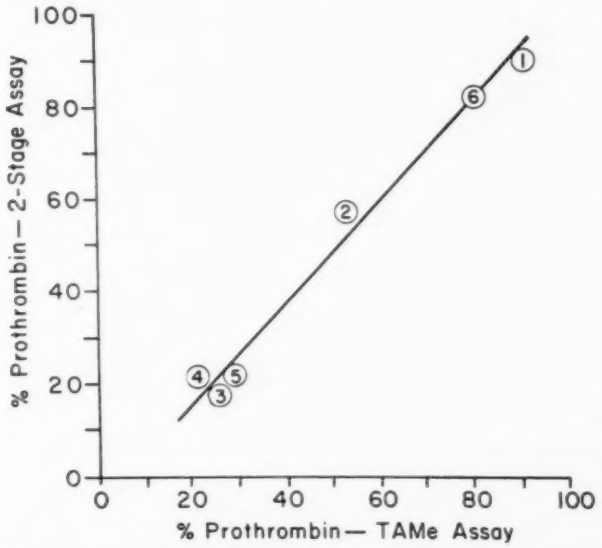

Fig. 2.—Comparison of TAMe and two-stage prothrombin assay results. Each of the 6 points represents the average of 20 TAMe assays plotted against the average of the corresponding 20 two-stage determinations. For this comparison, randomly selected data from patients on anticoagulant therapy were used at the designated points: (1) Normal, pretherapy assay; (2) Day 1 after start of therapy; (3) Day 1 at control level; (4) Day 2 at control level; (5) Day 1 after removal of therapy; (6) Last analysis.

This value compares favorably with the average of 53 units reported for normal plasmas by Glueck, Sherry, and Troll. The results plotted in Fig. 2 indicate that at all other two-stage prothrombin levels the TAMe assay produces a corresponding number of TAMe units. Statistical treatment of these data shows that at all but normal prothrombin levels there are significant differences in the results of the two assays ($P = 0.05$). This finding is not surprising in view of the fundamental differences in the two tests and indicates that, strictly speaking, they are measuring different entities probably resulting from the variable manner in which prothrombin is activated. For practical purposes, however, it is apparent that both techniques do measure similar levels of activity (Fig. 3). Added support for this conclusion is provided by the good agreement shown between the results of the two assay methods when applied to the plasmas of patients maintained on any of the 8 prothrombin-depressant drugs studied (Fig. 4). Data similar to these have also been reported recently by Glueck.[15] It is apparent that the characteristics of the particular drug used do not significantly affect the TAMe-two-stage relationship at any plasma prothrombin concentration.

Effect of Thromboplastin.—The kind of tissue thromboplastin used in the TAMe assay was originally thought to have no influence on the outcome of this determination since identical results were obtained when normal human plasmas were assayed with any 1 of 4 thromboplastins commercially available* and 3 laboratory-prepared lipoprotein thromboplastins (Table I).[16] Additional support

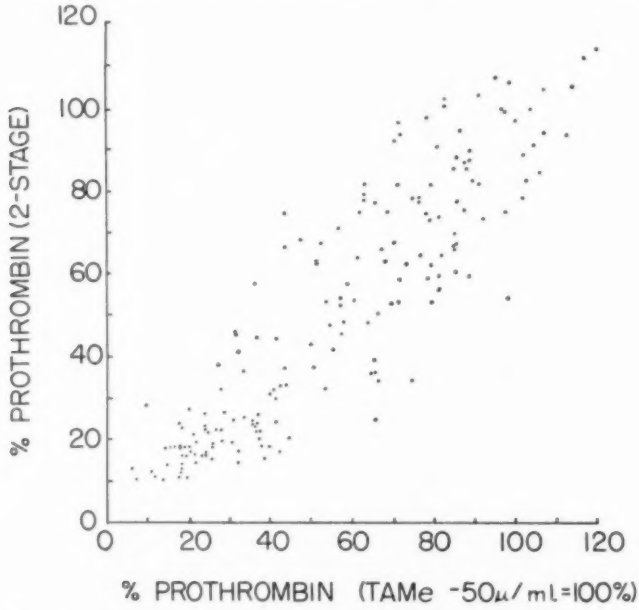

Fig. 3.—Plasma prothrombin levels of normal and therapy patients assayed simultaneously by the two-stage and TAMe methods.

for this preliminary conclusion was provided by the observation that saline dilutions of normal human plasmas to levels as low as 10 per cent of normal each assayed to the same number of TAMe units regardless of which of 3 commercial thromboplastins were used to activate prothrombin (Table I). Similar results were also obtained for plasmas from patients on anticoagulant therapy as long as their two-stage prothrombin levels were between normal (80 to 100 per cent) and the upper limit of the control bracket. However, with therapy plasmas having two-stage prothrombin levels below 30 per cent of normal the TAMe assay results varied according to the nature of the thromboplastin used. (Table I and Fig. 5.) Since these variations were only observed at low prothrombin levels resulting from drug therapy, but not in the case of those resulting from dilutions of normal plasmas, it was concluded that some other factor or factors, also depressed by anticoagulants, might be supplied to the assay

*Supplied through the courtesy of Difco Laboratories, Detroit, Mich.; Geigy Company, Inc., New York, N. Y.; Schieffelin & Company, New York, N. Y.; and Warner-Chilcott Laboratories, New York, N. Y.

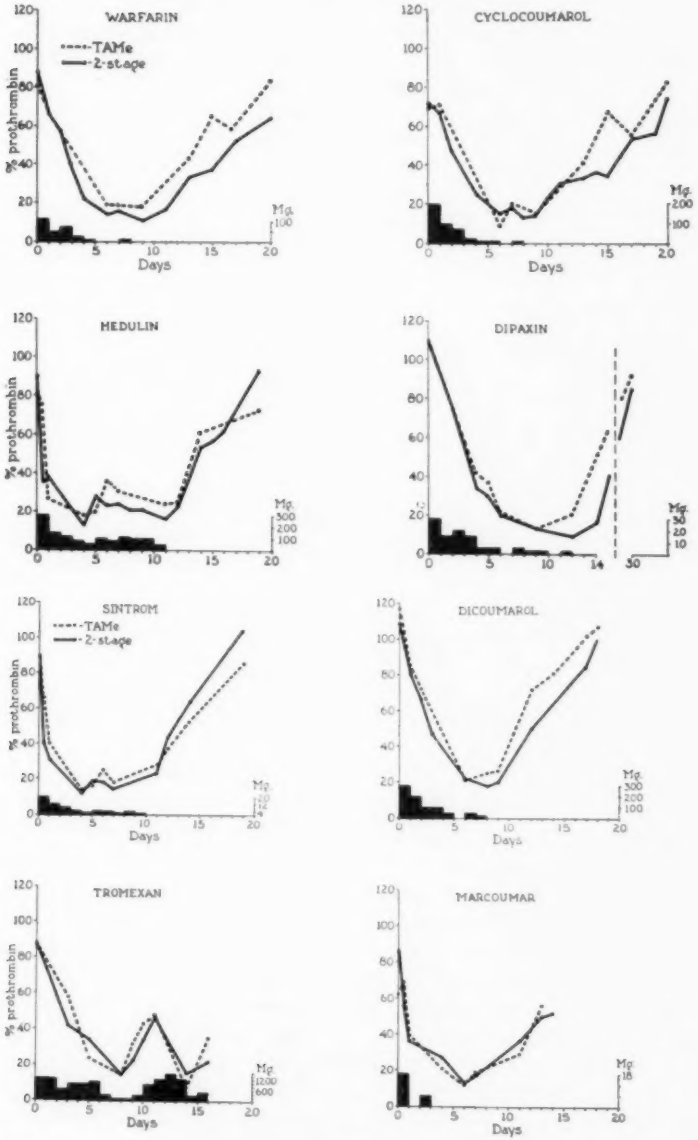

Fig. 4.—Two-stage and TAMe assay results: patients on 8 different anticoagulants.

system in varying concentrations by the different thromboplastins used. Such factors presumably would have to decrease more rapidly as a result of therapy than prothrombin itself and, theoretically at least, their concentration at very low levels might limit the extent of prothrombin conversion possible during a fixed period of time (such as the 5 minute activation period used in the TAMe assay). Factor VII (SPCA) has been reported to decline more rapidly than

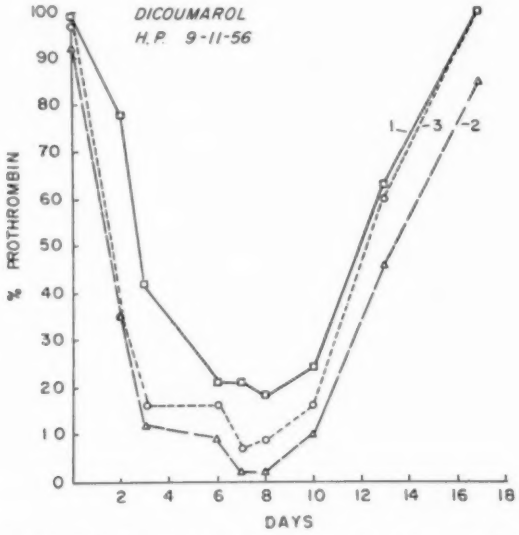

Fig. 5.—Variations in TAMe prothrombin curves in a single patient with use of 3 different thromboplastins. The thromboplastins used were: (1) Difco Bacto-Thromboplastin; (2) Schieffelin Soluplastin; and (3) Warner-Chilcott Simplastin.

prothrombin as a result of therapy with some anticoagulants[17]; some information is available concerning the *rate of decline* of Factor IX (PTC)[17] but, to our knowledge, none concerning that of Factor X. The possibility arose that different thromboplastin preparations might contain different amounts of Factor VII and in this way produce different results in the TAMe assay with low-level therapy plasmas. Furthermore, since Ac-globulin activity has been found to be associated with certain thromboplastins[18, 19] and since the effective Ac-globulin activity of a preparation is dependent upon a minimum Factor VII level,[21] varying Ac-globulin levels were also considered as a possible cause of the variations in the TAMe assay results obtained with low-level therapy plasmas and different thromboplastins. Preliminary findings (Table II) in our laboratory, that the addition of purified Factor VII and/or Ac-globulin raised the activities of the less active thromboplastins to the level of the most active one, suggest that these factors may be present to varying degrees in different thromboplastins and, therefore, responsible for the variations in TAMe assay results obtained with low-level therapy plasmas.[20]

TABLE I. TAMe PROTHROMBIN PERCENTAGES (50 TAMe UNITS = 100 PER CENT PROTHROMBIN) RESULTING FROM THE USE OF DIFFERENT THROMBOPLASTINS. EACH FIGURE REPRESENTS THE AVERAGE OF AT LEAST THREE EXPERIMENTS

	PER CENT PROTHROMBIN (TAMe)		
THROMBOPLASTIN	NORMAL PLASMA (2-STAGES: 80-100 PER CENT)	NORMAL PLASMA (DILUTED 4 TIMES) (2-STAGES: 20-25 PER CENT)	THERAPY PLASMA (2-STAGES: <30 PER CENT)
Bacto-Thromboplastin (Difco)	96	24	18
Simplastin (Warner-Chilcott)	92	23	13
Soluplasin (Schieffelin)	94	24	8
Geigy thromboplastin	98		6
Human brain	94		3
Beef brain	94		9
Beef lung	92		12

TABLE II. THE EFFECT OF ADDITION OF FACTORS V AND VII ON PROTHROMBIN PERCENTAGES (TAMe) OF THERAPY PLASMAS

	THERAPY PLASMA PLUS SUPPLEMENTED FACTORS PER CENT PROTHROMBIN (TAMe)			
THROMBOPLASTIN	NO SUPPLEMENT	WITH FACTOR V (FINAL CONCENTRATION = 1:25)	WITH FACTOR VII (FINAL CONCENTRATION = 1:100)	WITH FACTORS V + VII
A. Bacto-Thromboplastin (Difco)				
Experiment 1	12	11	11	11
Experiment 2	20	18	25	25
Experiment 3	22	20	18	19
B. Bacto-Thromboplastin (Difco)	18	15	17	18
Simplastin (Warner-Chilcott)	13	17	17	18
Soluplastin (Schieffelin)	8	7	21	22

Factor supplements were added to the assay just prior to the addition of plasma and the subsequent start of activation. Figures recorded under A are the results of three experiments performed on different days (3 plasmas) with thromboplastin prepared daily from different batches of Bacto-Thromboplastin (Difco). The results of experiment 2 indicate that variations may occur even with a thromboplastin relatively high in factor content. Each figure recorded under B is the average of at least 6 experiments.

DISCUSSION

It is apparent that the main differences of opinion concerning the efficacy and safety of anticoagulant drugs are related to the methods used to control their administration. In the midst of the controversy over which and whose method is "best," the main issue of clinical concern, namely, the question of what actually should be measured in order to control therapy, is often obscured. Since the primary purpose of anticoagulant therapy is to reduce the tendency of blood to clot, it would appear that the ideal control of such therapy should be based on a method for determining the over-all result of the effects of the drug on prothrombin itself, various conversion accelerators, and possibly inhibitors. The measure of this over-all activity might be called the "effective thrombic activity" of a patient's plasma and will be influenced by variations in a number of different clotting factors. These individual factor variations themselves may depend not only on the particular drug used but may, for any given drug, actually differ from patient to patient.[22]

With regard to the type of thromboplastin used in the TAMe assay we know that of the commonly used preparations some are more deficient in certain accelerators than others, and we might add that they may also vary in

their content of inhibitors. Of the available thromboplastins those having the greater deficiencies in accelerators would appear to be the more preferable ones to use from the point of view of estimating "effective thrombic activity" in therapy plasmas. The possibility of preparing a lipoprotein-type thromboplastin with characteristics even more ideal in reference to accelerator and inhibitor content is suggested by the work of Nour-Eldin and Wilkinson.[23] The use of such a preparation would allow the most accurate determination of a plasma's "effective thrombic activity."

The bulk of the data reported in this paper was obtained with a commercial rabbit brain thromboplastin which is not as free of Factors V and VII as some of the other preparations tested. In spite of the differences in technique between the two-stage and TAMe assays it is surprising that fairly good agreement between the two methods was obtained even at control levels. It would appear that the variable contributions of accelerators and/or inhibitors made by the beef lung thromboplastin used in the two-stage method together with the variable periods of prothrombin activation employed in this assay combine or complement each other somehow to yield thrombin activities similar to those obtained under quite different conditions in the TAMe assay.

SUMMARY

1. Two-stage and TAMe prothrombin determinations have been carried out on plasmas from patients on 8 different anticoagulant drugs. For clinical purposes, the results obtained with the TAMe assay have been found to compare favorably with those provided by the two-stage technique regardless of which of the 8 drugs was used.

2. The concept of "effective thrombic activity" has been introduced and discussed in regard to the possible accelerator and/or inhibitor content of the thromboplastin preparation used in the TAMe assay.

REFERENCES

1. Quick, A. J., Stanley-Brown, Margaret, and Bancroft, F. W.: A Study of the Coagulation Defect in Hemophilia and in Jaundice, Am. J. Med. Sc. **190**: 501-511, 1935.
2. Ware, A. G., and Seegers, W. H.: Two-Stage Procedure for the Quantitative Determination of Prothrombin Concentration, Am. J. Clin. Path. **19**: 471-487, 1949.
3. Biggs, Rosemary, Douglas, A. S., and Macfarlane, R. G.: Action of Thromboplastic Substances, J. Physiol. **122**: 554-569, 1953.
4. Owen, Charles A., Jr., and Bollman, Jesse L.: Analysis of the One-Stage Test for Prothrombin Time, J. Appl. Physiol. **5**: 515-525, 1953.
5. Ferguson, John H., and Patch, Mary Jane: Determinants of the So-called "Prothrobin Time," Proc. Soc. Exper. Biol. & Med. **93**: 193-196, 1956.
6. Ferguson, John H., Lewis, Jessica H., and Fresh, James W.: The Two-Stage "Prothrombin" Assay in Study of Bleeding and Clotting Disorders, Yale J. Biol. & Med. **28**: 253-264, 1956.
7. Olwin, J. H.: Methods for Prothrombin Assay. Theoretical and Practical Considerations, Schweiz. med. Wchnschr. **29**: 817-828, 1954.
8. Sherry, S., and Troll, W. J.: The Action of Thrombin on Synthetic Substrates, J. Biol. Chem. **208**: 95-105, 1954.
9. Glueck, Helen I., Sherry, Sol, and Troll, Walter: Assay of Plasma Prothrombin With a Synthetic Substrate, Proc. Soc. Exper. Biol. & Med. **87**: 646-650, 1954.
10. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: A Quantitative Study on Blood Clotting: Prothrombin Fluctuations Under Experimental Conditions, Am. J. Physiol. **114**: 667-675, 1936.

11. Lewis, M. L., and Ware, A. G.: A Simple Procedure for Separation of Prothrombin and Accelerator Globulin From Citrated Human Plasma, Proc. Soc. Exper. Biol. & Med. **84**: 636-640, 1953.
12. Alexander, Benjamin, Goldstein, Robert, and Landwehr, Greta: The Prothrombin Conversion Accelerator of Serum (SPCA): Its Partial Purification and its Properties Compared With Serum Ac-Globulin, J. Clin. Invest. **29**: 881-895, 1950.
13. De Nicola, Pietro: The Laboratory Diagnosis of Coagulation Defects, Charles C Thomas, Springfield, Ill., 1956, p. 158.
14. Biggs, Rosemary, and Macfarlane, R. G.: Human Blood Coagulation and Its Disorders, Charles C Thomas, Springfield, Ill., 1953, p. 344.
15. Glueck, Helen I.: The Utilization of a Synthetic Substrate (TAMe) to Measure the Plasma Prothrombin in Coagulation Disorders, J. Lab. & Clin. Med. **49**: 41-60, 1957.
16. Arscott, Phyllis M., Koppel, J. L., and Olwin, John H.: Advantages of TAMe Assay for Routine Determinations of Plasma Prothrombin, Fed. Proc. **15**: 506, 1956.
17. Koller, F., Loeliger, A., and Duckert, F.: Experiments on a New Clotting Factor (Factor VII), Acta haemat. **6**: 1-18, 1951.
18. Johnson, S. A., Seegers, W. H., Koppel, J. L., and Olwin, J. H.: The Reduction of Platelet Cofactor II (Autoprothrombin II) Activity with Anticoagulants, Thrombosis et Diathesis, Hemorrhagica **1**: 158-168, 1957.
19. Verstraete, Marc, Clark, Patricia, A., and Wright, Irving S.: Use of Different Tissue Thromboplastins in the Control of Anticoagulant Therapy, Circulation **16**: 213-226, 1957.
20. Koppel, J. L., Arscott, P. M., Friedman, L. H., and Olwin, J. H.: Differences in Tissue Thromboplastins and Their Influence on the Control of Anticoagulant Therapy, Surgery **43**: 94-101, 1958.
21. Koppel, J. L., Mueller, Dorothy, and Olwin, John H.: Nature of the Inhibition of Thrombin Formation by Sulfhydryl-Oxidizing Agents, Am. J. Physiol. **187**: 113-121, 1956.
22. Dionysios, Adamis, Sise, Herbert S., and Kimball, Delbert, M.: The Proconvertin Test: A Simplified Method and its Application to the Study of Anticoagulant Processes, J. Lab. & Clin. Med. **47**: 320-325, 1956.
23. Nour-Eldin, F., and Wilkinson, John F.: The Separation of Human and Bovine Plasma Thromboplastin With Ether and a Study of its Properties, J. Physiol. **132**: 164-172, 1956.

THE USE OF CHICKEN ANTISERUM FOR THE RAPID DETERMINATION OF PLASMA PROTEIN COMPONENTS

III. The Assay of Human Serum Transferrin

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OUR previous work[1] demonstrated a close correspondence between immunochemical and electrophoretic determinations of human serum albumin and gamma globulin. Similarly the immunochemical assay of orosomucoid[2, 3] accorded with chemical estimation of the serum mucoprotein fraction. The present study compares the transferrin and total iron binding capacity of human serum and again demonstrates the utility of chicken antiserum in the determination of protein.

Transferrin (or siderophilin) is a beta-1 protein, devoid of lipid, low in carbohydrate, and possessing a molecular weight of about 90,000.[4-6] It has striking metal combining properties and appears to be the sole protein responsible for the transport of serum iron.[4, 5, 7] The isolation and characterization of transferrin have been described by Surgenor and associates[4, 5] and Koechlin.[6] Crystalline preparations were injected into rabbits by Jager and Gubler[8]; a number of antisera were produced, but these yielded variable results when used for assay purposes. Inman[9, 10] modified Koechlin's fractionation procedure and has succeeded in regularly crystallizing the protein. The present study was carried out with one of Inman's crystalline preparations.

METHODS AND MATERIALS

Twice crystallized human serum transferrin was the immunizing antigen and the primary standard. By electrophoretic analysis* the preparation had a purity of over 97 per cent. We determined immunochemically[1] that it contained 0.5 per cent albumin and 0.2 per cent gamma globulin.

In all, 21 Rhode Island Red hens, 8 to 12 months of age, were immunized. A single intravenous injection of 40 mg. antigen per kilogram of body weight, according to the method of Abramoff and Wolfe,[11] invariably resulted in a positive precipitin response.

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Bleeding was 8 days after the injection. The procedure of intramuscular injection of the antigen (100 to 200 mg.) well mixed with $Al(OH)_3$ gel and with bleeding ten days later also consistently yielded positive sera. However, by either method the potencies of the antisera were quite variable and were much less than that afforded by similarly produced antialbumin and antigamma globulin sera.

In our initial studies the citrated chicken blood, obtained at the time of bleeding, was processed according to the method of Silberberg and co-workers.[2] Subsequently the processing was modified so that the antisera were at a final NaCl concentration of 13 per cent instead of 8 per cent.

Potency was estimated by the qualitative procedure employed in the orosomucoid study.[3] Oudin tests[12] were also performed as previously described.[1, 3] In these tests each antiserum was reacted against crystalline transferrin, human serum, and various Cohn fractions (II, III, IV-1, IV-4, and V).

An additional method for specificity analysis* was provided by the immunoelectrophoretic procedures developed by Poulik[13-15] in which the antigenic mixture is first separated by electrophoresis on starch gel[16] or filter paper. We found that to successfully examine chicken antiserum by this method a high salt concentration must be incorporated in the agar, and in the present study 13 per cent NaCl was employed.

The biuret determination of protein was carried out with the reagent of Gornall and associates.[17] The method of Peters and co-workers[18] was used to assay the total iron binding capacity (T.I.B.C.) of serum. The samples of human serum were largely obtained from the Oncology Service of Henry Ford Hospital.†

TABLE I. A COMPARISON OF THE REACTIONS OF ANTITRANSFERRIN SERUM WITH CRYSTALLINE TRANSFERRIN BEFORE AND AFTER ABSORPTION WITH GAMMA GLOBULIN

	OPTICAL DENSITY READINGS AT 450 mμ	
μg CRYST. TRANS.	UNABSORBED ANTISERUM	ABSORBED ANTISERUM
910	0.576	0.568
182	0.629	0.624
91	0.572	0.568
45.5	0.545	0.537
22.8	0.494	0.465
11.4	0.357	0.328
5.7	0.244	0.224
μg Fr II		
80	0.081	0.007
20	0.078	0.007
5	0.046	0.007

Each reaction tube contained 0.3 ml. antigen in 13 per cent NaCl and 0.2 ml. antiserum (282-84) at a dilution of 1:2.32 in 13 per cent NaCl. After 1 hour of incubation at 38° C., reaction turbidities were read in the Bausch and Lomb Spectronic 20.

RESULTS

Specificity Analyses.—The antisera were subjected to Oudin and immunoelectrophoretic analyses. Typically, as can be noted in Fig. 1, there was a strong transferrin reaction and a weak gamma globulin reaction. Reactions with other human serum proteins were never detected. In view of this, we thought that certain of the antibodies to transferrin might cross react with gamma globulin. This possibility was investigated by the absorption experiment presented in Table I. It can be seen that removing the antibodies to

*We wish to acknowledge the invaluable participation of Emily Poulik in this phase of our study.

†We are indebted to Dr. Michael J. Brennan for his assistance in providing us with sera from his patients.

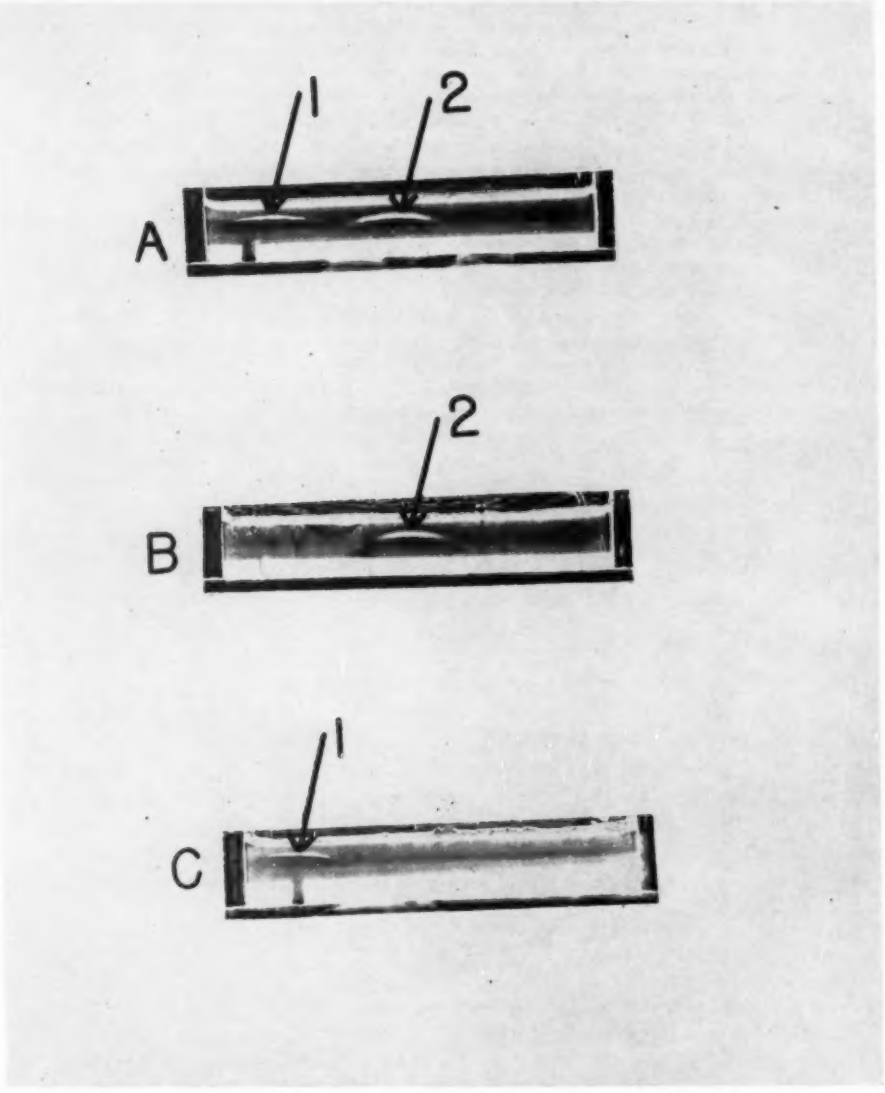

Fig. 1.—Specificity characteristics of antitransferrin serum (282-84) as visualized by starch gel immunoelectrophoresis.* Human serum constitutes the antigenic mixture. *A*, Unabsorbed. *B*, Absorbed with Fr II. *C*, Absorbed with crystalline transferrin. 1 = Precipitin line due to gamma globulin. 2 = Precipitin line due to transferrin. A nonspecific lateral band develops in the agar near the surface of the starch block in some immunoelectrophoresis runs with chicken antiserum. Since this band is readily recognized, it does not interfere with the determination of the number of antigen-antibody reaction arcs.

*Similar patterns were developed in paper strip immunoelectrophoresis, and the beta-1 location of the transferrin arc was clearly evident.

gamma globulin did not significantly diminish the precipitation of the antiserum with crystalline transferrin. Apparently, then, the small amount of gamma globulin present in the immunizing preparation was capable of eliciting an independent antibody response, even though each bird was injected with only about 0.25 mg. of the contaminant. Little role, if any, could be ascribed to hypothetical antitransferrin antibodies cross reacting with gamma globulin. Each 100 ml. of antiserum was absorbed with 10 mg. of Fr II before being used for assay purposes.

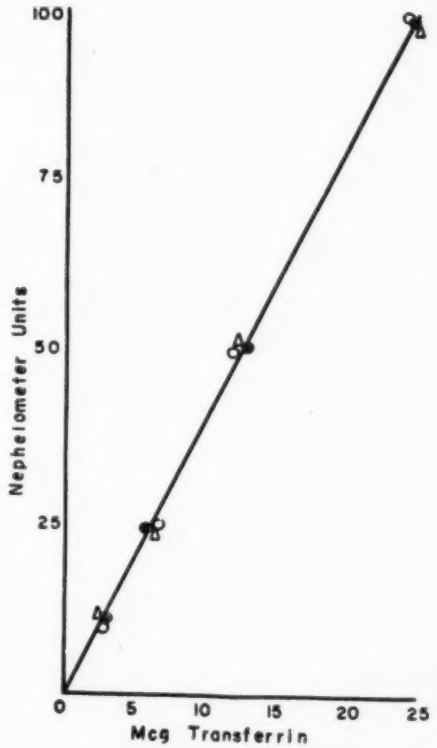

Fig. 2.—The standard curve in the determination of transferrin using the nephelometric method. Three successive determinations with P-29 are shown.

Nephelometric Assay Procedure.—The assay of human serum transferrin was carried out by the nephelometric procedure with our initially produced pool of antiserum (P-29). This pool was at the routine 1:2 dilution in 8 per cent NaCl. One-half milliliter of antigen solution, 4 ml. of diluent, and 1 ml. of P-29 were successively added to each reaction tube. The NaCl concentration was at 8 per cent and the incubation period was 20 minutes at 38° C. Standard curves were constructed with crystalline transferrin, ranging in

amount from 25 to 3.13 μg. The curve used in the assay of many of the human serum samples is presented in Fig. 2. The quantity of human serum employed in a determination was 0.005 ml. (0.5 ml. of 1:100 dilution). With this amount normals fell at the midportion of the curve, and pathologic samples, whether exhibiting depressed or elevated transferrin values, were readily encompassed by the standards. The micrograms determined to be in the sample aliquot were multiplied by 20 to convert to milligrams transferrin per 100 ml. of serum (mg. per cent).

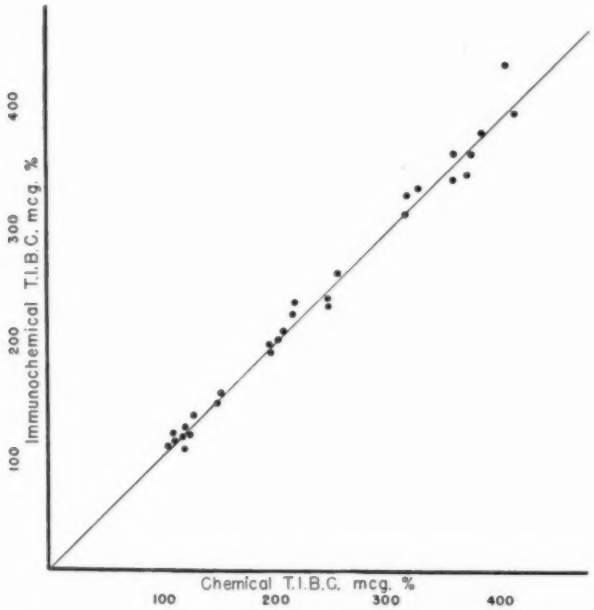

Fig. 3.—Identity of immunochemical and chemical determinations of total iron binding capacity (T.I.B.C.). The samples of human sera were drawn from patients with a variety of clinical syndromes and each point represents a different serum sample. Low values were generally from patients with malignancy, and high values were generally from obstetrical cases and patients with uncomplicated iron deficiency anemia.

The total iron binding capacities were determined on 29 of the samples which had been analyzed for transferrin by the immunonepholometric method. The results of the two sets of determinations are compared graphically in Fig. 3. The transferrin values were multiplied by 1.25 since each milligram of protein binds 1.25 μg of iron.[5] The identity of T.I.B.C. with transferrin concentration can be readily seen.

Spectrophotometric Assay Procedure.—The potency of the next batch of pooled antitransferrin serum, P-35, was weaker than that of P-29. P-35 was adjusted to 13 per cent NaCl, and the spectrophotometric procedure of assay[3] was employed. The reactions, each consisting of 0.1 ml. of antigen solution in

13 per cent NaCl thoroughly mixed with 0.4 ml. of P-35, were set up in 10 by 75 mm. Pyrex test tubes. After an incubation period of 1 hour at 38° C., the contents of each tube were shaken, poured into a suitable microcell,* and read in a spectrophotometer set to zero optical density with an antiserum reagent blank.

The pooled human serum (PCS) described in our previous publication[3] provided the routine standard for sample assay. A transferrin content for PCS of 184 mg. per cent was determined by the immunonephelometric procedure with P-29 and was found to agree with the T.I.B.C. of 232 μg per cent determined by the method of Peters and associates.[18] Aliquots of PCS employed for constructing the calibration curve contained, respectively, 9.2 μg., 4.5 μg, 2.25 μg, and 1.13 μg transferrin, and the fixed quantity of human serum for sample determination was 0.1 ml. of a 1:50 dilution. The micrograms of antigen determined to be in a simple aliquot were multiplied by 50 to convert to mg. per cent transferrin. When duplicate determinations were performed on 16 serum samples ranging from 113 to 400 mg. per cent transferrin the coefficient of variation was found to be ±8 per cent. In this experiment the 1:50 dilutions were made afresh for each set of determinations, and the repeat analyses on 6 of the samples were carried out on the following day.

We were particularly interested in comparing the calibration curves afforded by PCS and crystalline transferrin, inasmuch as it had previously been observed[3] that PCS of assigned orosomucoid value and the purified orosomucoid yielded slightly divergent curves with antiorosomucoid sera. Two of the antitransferrin sera also displayed a similar reactivity. As shown in Fig. 4, somewhat more precipitation was afforded by PCS than by crystalline transferrin in the far antibody excess zone. This divergence of the curves was reproduced on two separate determinations, each time employing fresh dilutions of the antigens. It is unlikely that the transferrin value assigned to PCS was fallacious, since it was independently determined by a precise chemical method.[18] There appears, therefore, to be a small but real difference in the immunologic behavior of PCS† and crystalline transferrin.

During the current studies four pools of antitransferrin serum have been employed. The sample assay results obtained with these antisera were found for practical purposes to be interchangeable. To establish this clearly, 11 samples, each a pool of serum from several bleedings of a single patient, were separately analyzed for transferrin content by the 4 antisera and were also analyzed for T.I.B.C. The close correspondence of results can be seen in Table II.

*Fused silica microcells, 3 by 10 by 25 mm., produced by Pyrocell (207 E. 84th Street, New York 28, New York) were used with the Beckman D.U. spectrophotometer at 340 mμ. The Arthur H. Thomas microadapter (no. 9085-D2) and absorption cell (9085-N2) were used with the Bausch and Lomb Spectronic 20 at 450 mμ. The minimum volume required for each microcell was no more than 0.4 ml. Comparable reaction optical densities were observed with both spectrophotometers at the above wave lengths.

†Since PCS largely represented pooled cancer serum we recently repeated the reactions with pooled normal serum (PNS). Again the calibration curves obtained with PNS and crystalline transferrin were divergent.

Levels of Transferrin in Human Serum.—The transferrin contents were determined immunochemically on sera from 47 normal individuals. Values ranged from 229 to 360 mg. per cent and the mean for the series was 281.7 mg. per cent (±8.06 mg. S.E.). These results when converted to T.I.B.C. by the factor 1.25 are in the range of the T.I.B.C. values for normals established by other investigators (see Peters and associates[18] for references). Our results also correspond to those of others in that we have observed elevated transferrin values in uncomplicated iron deficiency anemia and in pregnancy as well as depressed transferrin levels in infectious states and in malignancy.

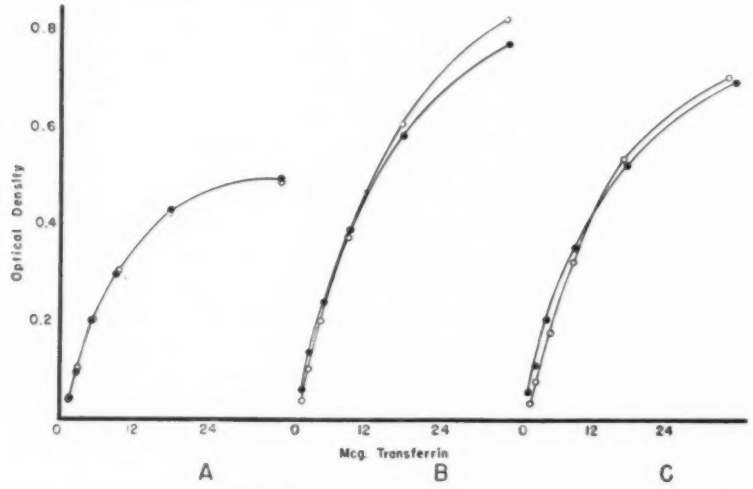

Fig. 4.—Comparison of antibody excess zone reactions afforded by calibrated PCS and crystalline transferrin with 3 different antitransferrin sera. Solid circle = calibrated PCS reactions; undilute PCS was considered to contain 184 mg. per cent transferrin (see text). Open circle = crystalline transferrin reactions. Each point represents the average of two determinations. *A*, 0.1 ml. Ag + 0.4 ml. Ab (P-35); 13 per cent NaCl; 1 hr. incubation at 38° C. *B*, 0.1 ml. Ag + 0.4 ml. Ab (P-39); 13 per cent NaCl; 1 hr. incubation at 38° C. *C*, 0.1 ml. Ag + 0.4 ml. Ab (1/2.32 absorbed 282-84); 13 per cent NaCl; 1 hr. incubation at 38° C. Reaction turbidities recorded in a Beckman spectrophotometer set at 340 mμ.

TABLE II. COMPARISON OF TRANSFERRIN VALUES OBTAINED BY FOUR DIFFERENT ANTISERA AND BY PETERS' METHOD

SAMPLE NO.	CHEM. T.I.B.C. (μg %)	CHEM. TRANS. (MG. %)	P-29 (MG. %)	P-35 (MG. %)	P-39 (MG. %)	282-84 (MG. %)
1	214	171	165	170	176	175
2	90	72	58	65	54	65
3	251	201	199	200	210	225
4	168	134	139	120	139	135
5	430	344	370	375	378	320
6	322	257	247	265	250	230
7	232	185	175	195	185	187
8	186	149	143	140	136	137
9	214	171	170	175	177	192
10	249	198	193	185	188	183
11	330	264	245	257	256	255

DISCUSSION

The antisera produced in chickens to single injections of Inman's crystalline transferrin[9, 10] were successfully used to measure the total iron binding capacity of human serum. In contrast to these results, Jager and Gubler[8] found that rabbits responded poorly to transferrin and yielded positive antisera only after prolonged immunization. Such antisera yielded variable assay results and apparently contained many more antibodies to contaminants than the chicken antisera employed by us. In this regard we analyzed rabbit antisera* to Inman's crystalline transferrin by immunoelectrophoresis and observed reactions with at least 5 different human proteins.

The accuracy of assay by the immunochemical method is not only dependent on the specificity of the antisera but on the type of standard employed. Typically, calibration curves are constructed from the immunizing antigen, and if the antisera produced to it fail to yield reproducible sample assays, the purity of the antigen is held to be inadequate. Further purification of the immunizing preparation will, in this event, undoubtedly lessen the occurrence of antibody responses to unrelated antigens. Yet a consideration of protein microheterogeneity[19] leads to the conclusion that even antisera produced to an antigen so purified that distinct contaminants are lacking could still yield variable sample determinations. From the concept reviewed by Colvin and coworkers[19] that a native protein species is a discrete population of closely related but not identical molecules, it follows that the exact distribution of the molecular forms of the species can rarely, if ever, be duplicated in the isolated product. Accordingly, the native protein in human serum and its isolated counterpart need not exhibit uniform immunologic reactivity, even with highly specific antisera. Furthermore, such antisera could contain dissimilar amounts of antibodies to the antigenic components. Our proposed solution[1] to the problem of obtaining comparative sample assay values of high accuracy has been to use pooled human serum, calibrated for the protein under determination, as the routine standard. Not only should such a standard tend to compensate for trace contaminant or undetermined cross reactions of the antisera (as previously pointed out[1]), but it should also tend to compensate for any variability in the reactions of the antisera with microheterogeneous components of the antigen.

The divergence of the calibration curves afforded by pooled human serum (PCS) of known iron binding capacity and crystalline transferrin with two of the antitransferrin sera (Fig. 4) serve to illustrate the above discussion. It was also observed (unpublished data) with each of these antisera that after an absorption with a slight excess of crystalline transferrin, antibodies remained which failed to precipitate with a small quantity of transferrin (e.g., 4.6 μg per 0.3 ml. supernatant) but did precipitate with a large quantity (91 μg per 0.3 ml. supernatant). These antibodies appeared to be directed to microheterogeneous transferrin components, rather than to contaminant antigen(s),

*We wish to thank M. D. Poulik for giving us portions of these antisera.

for the only precipitin line that they developed in immunoelectrophoresis was with the human serum component (beta-1) of highest transferrin concentration.

The assumption that microheterogeneity exists at the antigenic level, while by no means established by our observations, may lead to new approaches for detecting small, but biologically significant, protein differences. We have in mind such differences in the protein products of cells as might occur at various stages of tissue morphogenesis or might arise from mutations of either the soma or germ plasm. The molecular forms comprising a protein family would be expected to have a set of common antigenic determinants and a number of dissimilar ones with representatives of the latter occupying only a few of the surface sites of an average molecule. This type of antigenic variability, if it exists, should not markedly affect the quantitative determination of the individual protein families by specific antisera, particularly when standards of pooled human serum are used. In this regard, we have demonstrated that the characteristic variations of the total iron binding capacity of human serum, such as its increase in uncomplicated iron deficiency anemia and its decrease in advanced malignancy, are accurately reflected by the transferrin levels determined immunochemically.

SUMMARY

Specific antihuman transferrin serum was readily produced in chickens by single antigen injection procedures. This was contrasted to the difficulty experienced by others in producing a comparable reagent in rabbits. The chicken antisera were successfully used to assay the transferrin concentration of human serum. Microquantities of sample were chosen, and rapid turbidimetric procedures of assay were employed. The immunochemical determinations agreed with chemical estimations of total iron binding capacity, and the mean serum transferrin level of normal adults was found to be 281.7 mg. per cent (±8.06 mg. S.E.). The possible effects of protein microheterogeneity on the accuracy of immunochemical determinations were discussed.

REFERENCES

1. Goodman, M., Ramsey, D. S., Simpson, W. L., Remp, D. G., Basinski, D. H., and Brennan, M. J.: The Use of Chicken Antiserum for the Rapid Determination of Plasma Protein Components. I. The Assay of Human Serum Albumin and Gamma Globulin, J. LAB. & CLIN. MED. **49**: 151, 1957.
2. Silberberg, S., Goodman, M., Kefalides, N. A., and Winzler, R. J.: Immunochemical Determination of Orosomucoid, Proc. Soc. Exper. Biol. & Med. **90**: 641, 1955.
3. Goodman, Morris, Ramsey, David S., Simpson, William L., and Brennan, Michael J.: The Use of Chicken Antiserum for the Rapid Determination of Plasma Protein Components. II. The Assay of Human Serum Orosomucoid, J. LAB. & CLIN. MED. **50**: 758, 1957.
4. Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S., Jr., and Gibson, D. M.: The Separation of Choline Esterase, Mucoprotein, and Metal-Combining Protein into Subfractions of Human Plasma, J. Am. Chem. Soc. **71**: 1223, 1949.
5. Surgenor, D. M., Koechlin, B. A., and Strong, L. E.: Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXVII. Metal-Combining Globulin of Human Plasma, J. Clin. Invest. **28**: 73, 1949.
6. Koechlin, B. A.: Preparation and Properties of Serum and Plasma Proteins. XXVIII. The B. Metal-Combining Protein of Human Plasma, J. Am. Chem. Soc. **74**: 2649, 1952.

7. Schade, A. L., and Caroline, L.: Iron-binding Component in Human Blood Plasma, Science **104**: 340, 1946.
8. Jager, B. V., and Gubler, C. J.: An Immunologic Study of the Iron Binding Protein, J. Immunol. **69**: 311, 1952.
9. Inman, J. K.: Studies on the Purification and Properties of Human B_1-Metal-Combining Protein. Doctoral Thesis, Harvard University, 1956.
10. Inman, J. K.: Purification of Metal-Combining Protein From Fraction IV-7. Proceedings of the 10th Conference on the Plasma Proteins and Cellular Elements of the Blood, Cambridge, 1956, p. 46-53, Protein Foundation, Inc.
11. Abramoff, P., and Wolfe, H. R.: Precipitin Production in Chickens. XIII. A Quantitative Study of the Effect of Simultaneous Injection of Two Antigens, J. Immunol. **77**: 94, 1956.
12. Oudin, J.: Methode d'analyse immunochimique par precipitation specifique en milieu gelifie, Compt. rend. Acad. sc. **222**: 115, 1946.
13. Poulik, M. D.: Filter Paper Electrophoresis of Purified Diphtheria Toxoid, Canad. J. M. Sc. **30**: 417, 1952.
14. Poulik, M. D.: Tests of Purity of Diphtheria Toxins by Electrophoresis in Starch Gel, Nature **177**: 982, 1956.
15. Poulik, M. D.: Immunoelectrophoresis in Starch Gel. I. Description of the Method. (In preparation.)
16. Smithies, O.: Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults, Biochem. J. **61**: 629, 1955.
17. Gornall, A. G., Bardawill, C. J., and David, M. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177**: 751, 1949.
18. Peters, T., Giovanniello, T. J., Apt, L., and Ross, J. F.: A New Method for the Determination of Serum Iron Binding Capacity. I., J. LAB. & CLIN. MED. **48**: 274, 1956.
19. Colvin, J. R., Smith, D. B., and Cook, W. H.: The Microheterogeneity of Proteins, Chem. Rev. **54**: 687, 1954.

THE DETERMINATION OF KETONE BODIES IN BIOLOGIC FLUIDS

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THE existing methods for the determination of ketone bodies in biologic fluid have been summarized in several publications.[1-3] The difficulties of the existing methods have been evidenced by the failure to have developed a satisfactory procedure for routine clinical studies. The available methods depend upon the distillation[1] or the extraction[2] of acetone with subsequent measurement of the acetone by titrimetric[1] or colorimetric procedure.[2, 3] Although recent methods have good sensitivity, all of them are complicated and time consuming. This paper presents a simple, sensitive, rapid, and accurate method for the determination of acetone, acetoacetic acid, and β-hydroxybutyric acid in biologic fluids. In the method to be presented, acetone is distilled from the heated to the cooled end of a sealed tube and trapped in alkaline salicylaldehyde. The entire procedure can be completed in 2 hours as well as determining from 1 to 8 μg accurately.

MATERIALS AND METHODS

One per cent salicylaldehyde† in 3 N KOH is the trapping and color reagent, made up fresh for each set of determinations. The other reagents used in the test are: approximately 4 N H_2SO_4, 20 N H_2SO_4, saturated KOH, and 0.3 per cent K_2Cr_2O in water. The unit employed in this experiment is a Thunberg tube with a coinciding hole in the top of the tube and in the ground-glass joint. A distillation apparatus (Fig. 1) makes it possible to place one end of the Thunberg tube in boiling water and the boot or side arm of the top is placed in cold running tap water. This apparatus may be heated either by means of a gas flame or internally by means of an electric coil which is properly insulated.

Protein-free filtrates are prepared by the method of Somogyi[4] in a screw-cap test tube which is centrifuged 1,500 r.p.m. for 10 to 15 minutes. The filtrate is diluted so that each aliquot contains between 1 and 8 μg of acetone equivalent per milliliter. The stoppers of the Thunberg tubes are lubricated with a minimal amount of silicone grease and then inverted in a rack. One-half milliliter of 1 per cent salicylaldehyde in 3 N KOH is added to the side arm or top of the Thunberg tube. One milliliter of the sample to be tested, or distilled water for a blank, is placed in the bottom of the Thunberg tube. One milliliter of 4 N H_2SO_4 is then added. The top is then carefully inverted so as not to spill the color reagent into the tube. The top is placed securely on the tube and fastened with adhesive tape. For the determination of acetone and acetoacetic acid, the tubes are heated for 90 minutes in the distillation apparatus shaking the tubes gently each 15 minutes, being careful to avoid spilling the color reagent into the bottom of the tube. For the determination of total

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†Eastman Kodak Company, Rochester, N. Y.

ketone bodies, the tubes are heated for 15 minutes, removed from the bath for 5 minutes, and then 1 ml. of acid dichromate reagent is added from a tuberculin syringe by approximating the coinciding holes in the stopper and the top of the tube. This step is done rapidly to avoid the possible loss of any acetone despite the fact that the cooling tube has a negative pressure during this period. The stopper is then turned to seal off the tube, the adhesive tape is reapplied, and the tube is returned to the distillation apparatus for 90 minutes; gently agitate the tubes at 15-minute intervals. The tubes are then removed from the water bath, the tops immediately removed, placed on a test tube rack, and 1 ml. of saturated KOH is added to each side arm. The color reaction is allowed to develop for 30 minutes. Then 1 ml. of water is added to each tube, the solutions thoroughly agitated and then transferred to cuvettes and read at 470 mμ on a Coleman Jr. spectrophotometer. The reagent blank is set at 100 per cent transmission.

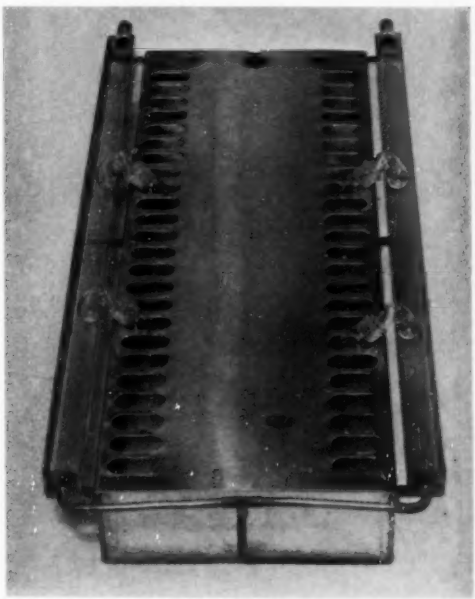

Fig. 1.—Distillation apparatus for the determination of ketone bodies showing Thunberg tubes in place. The hole in the ground-glass joint for the addition of chemicals is shown. Cold water is circulated through the side troughs and the main water bath is kept boiling.

RESULTS

The described method provides a linear relationship between the optical density of disalysalaldehyde acetone and the concentration of acetone in a range of 1 to 8 μg. Fig. 2 shows the results with the mean and standard error of an analysis of 21 standard curves run over a 6-month period. In this time, the maximum variation in standard readings was 10 per cent but good linearity was found for any one series (within 6 per cent). The time development curve of disalysalicylacetone at room temperature when saturated KOH is added shows that at 4 and 8 μg concentrations a plateau is reached

at 30 minutes and the color development is little changed for the next 30 minutes permitting ample time for spectrophotometric reading (Fig. 3). Analysis of random duplicates of 40 different plasma values ranging from 0.5 to 15 mg. per cent indicated a reproducibilty of 4.6 per cent in duplicate readings.

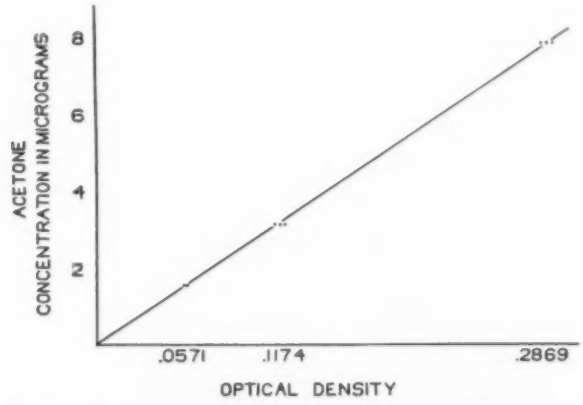

Fig. 2.—Composite acetone standard curve representing the color produced with alkaline salicylaldehyde against a blank. The curve is composed of 21 sets of determinations at 3 concentrations. The mean and standard error for each concentration is presented.

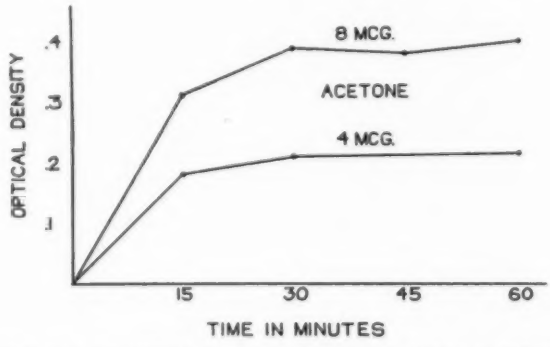

Fig. 3.—Curve of color development of the disalicylaldehyde acetone in strong alkali in respect to time. Two different acetone concentrations are shown.

Since the β-hydroxybutyric acid molecule must be converted to acetoacetic acid and then to acetone, recovery of this material reflects recovery of the other two materials as well. Table I presents the recovery of β-hydroxybutyric acid from 11 different plasma samples with concentrations ranging from 0.72 to 3.94 mg. per cent. Three different β-hydroxybutyric acid concentrations were added to each plasma sample prior to preparation of the filtrate and the results were calculated on the basis of amount found/amount expected times 100. Acetone standards were used in these studies and the results are expressed

as recovery in acetone equivalents. Twenty-six recoveries showed a level of accuracy of 94.5 ± 3.69 per cent which compares favorably with previous methods.

TABLE I. β-HYDROXYBUTYRIC ACID RECOVERIES FROM PLASMA IN MICROGRAMS OF ACETONE EQUIVALENTS

PLASMA CONC.	ADDED	EXPECTED	FOUND	% RECOVERED	PLASMA CONC.	ADDED	EXPECTED	FOUND	% RECOVERED
1.28	1.67	2.95	2.61	0.89	1.80	1.50	3.30	3.36	1.02
	3.33	4.61	4.49	0.97		3.0	4.80	4.29	0.89
	6.67	7.95	7.87	0.99		6.0	7.80	6.48	0.83
0.93	1.67	2.60	2.31	0.89	2.59	1.5	4.09	3.82	0.93
	3.33	4.26	4.01	0.94		3.0	5.59	5.22	0.93
	6.67	7.60	6.87	0.90		6.0	8.59	7.30	0.85
3.94	1.67	5.61	5.51	0.98	2.88	1.50	4.38	3.99	0.91
	3.33	7.27	6.81	0.94		3.0	5.88	5.82	0.99
						6.0	8.88	8.47	0.96
2.12	1.67	3.79	3.77	0.99	0.72	1.25	1.97	1.93	0.98
	3.33	5.45	4.98	0.91		2.50	3.22	2.94	0.92
	6.67	8.79	8.12	0.92		5.0	5.72	5.03	0.88
1.56	1.50	3.06	3.09	1.01	1.03	1.25	2.28	2.16	0.95
	3.0	4.56	4.36	0.95		2.50	3.53	3.11	0.88
	6.0	7.56	6.78	0.90		5.0	6.03	5.36	0.89
1.33									
	1.50	2.83	3.30	1.17					Mean 94.5 ± 3.69 S.D.
	3.0	4.33	4.48	1.06					
	6.0	7.33	7.56	1.03					

As the blood filtrate contains glucose which may vary considerably with the nutritional state, it was necessary to study the effects of varying glucose concentration on the recovery of β-hydroxybutyric acid. Dextrose concentrations of 100 to 500 mg. per cent were simulated in β-hydroxybutyric acid standards and the recovery of acetone equivalents was unaffected by glucose concentrations of 100 and 200 mg. per cent; above this level recovery decreases and desaccharification[1] may be necessary if the filtrate is not considerably diluted as a result of a high ketone concentration.

SUMMARY AND CONCLUSIONS

This method combines the use of a well-established color reagent for acetone (salicylaldehyde) with a new method of distillation and trapping of acetone. In the described procedure acetone is trapped in alkaline salicylaldehyde. Although some color development occurs during this period, it is kept at a minimum by cooling. The color development is brought about by adding saturated alkali to the alkaline salicylaldehyde acetone mixture. This last step is similar to that of Behre[5] who found that the heat resulting from adding saturated alkali was adequate for maximum color development in 20 minutes. The findings show that in the presented method, 30 minutes is the optimal color development time. Undoubtedly, distillation of acetone in the closed system presented in this method has advantages of avoiding loss of this volatile substance as well as making the whole procedure a one-unit operation.

The sensitivity of this method equals that of the most recent modification of the Greenberg and Lester method[2] and it exceeds the sensitivity of the previous salicylaldehyde methods.[3, 5] The reproducibility of the method is satisfactory as demonstrated by the results of this work. Although there is some variation from day to day in the standard curve, the relationship of concentration to optical density remains linear. The recovery of β-hydroxybutyric acid from plasma samples exceeds that reported by previous authors[5] using salicylaldehyde, indicating that this method is superior to those previously reported and is comparable to the most recent modification of the Greenberg and Lester method.[2]

The major advantage of the reported method is the feasibility of using it for routine clinical studies. This one point will make it possible to study the importance of using ketone body levels in the evaluation of patient care. Furthermore, the sensitivity of the method provides opportunities to study the fed as well as fasted patient, whereas previously, the sensitivity of methods more or less limited them to use in fasted patients in whom ketone bodies were elevated above normal.

The author would like to express his gratitude to Dr. Jane Russell for her advice during the development of this method, to Dr. John Lyon, with whom the distillation apparatus was developed in its present form, and to Miss Gloria Johnston, Mrs. Toh-Bee Syn, and Mrs. Betty Neill for their technical assistance.

REFERENCES

1. Weichselbaum, T. E., and Somogyi, M.: A Method for the Determination of Small Amounts of Ketone Bodies, J. Biol. Chem. **140:** 5, 1941.
2. Greenberg, L. A., and Lester, D.: A Micro-method for the Determination of Acetone and Ketone Bodies, J. Biol. Chem. **154:** 177, 1944.
3. Thin, C., and Robertson, A.: Estimation of Acetone Bodies, Biochem. J. **51:** 218-223, 1952.
4. Somogyi, M.: Determination of Blood Sugar, J. Biol. Chem. **160:** 69, 1945.
5. Behre, J. A.: A Modification of the Salicylaldehyde Method for the Determination of Acetone Bodies in Blood and Urine, J. Biol. Chem. **136:** 25, 1940.
6. Wirk, E. E., McPherson, H. T., Todd, H. W., Myers, J. D., and Engel, F. L.: Studies on the Ketone Metabolism in Man. I. A Method for the Quantative Estimation of Splanchnic Ketone Production, J. Clin. Invest. **34:** 1256-1267, 1955.

AN IMPROVED METHOD OF PEROXIDASE REACTION COMBINED WITH GIEMSA'S STAIN FOR BLOOD CELLS

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IN THIS report a simple method is described which demonstrates peroxidase activity clearly and also preserves the usual morphology of blood cells. This modification is compared with other widely utilized methods and its diagnostic value assessed.

Granulocytic cytoplasmic granules exhibit strongly positive oxidase or peroxidase activity, monocytic granules show a lesser degree of activity, whereas other bone marrow and peripheral leukocytes show no such cytoplasmic enzymatic activity.

Many modifications of the original indophenol-blue reaction (oxidase reaction) as described by Winkler[1] and by Schultze[2] have been utilized. Because of technical difficulties, including reagent instability, the most widely utilized techniques are those which employ the peroxidase reaction rather than the oxidase reaction. In our hands, these methods have been unsatisfactory because of the distortion of red cells, the obliteration of leukocytic detail and, more seriously, the failure of similar cells to yield similar peroxidase activity.

METHODS

Reagents.—(1) One part of 40 per cent formalin to 9 parts of 95 per cent ethanol. (2) Giemsa's solution. (3) Phosphate buffer solution, pH 6.4, N/15. (4) Five-tenths per cent $CuSO_4 \cdot 5\ H_2O$ solution. (5) One-tenth per cent benzidine solution (with the addition of 2 drops of 3 per cent H_2O_2 per 100 ml.).

The benzidine solution is prepared by slow trituration of 0.2 Gm. benzidine crystals (reagent grade) in a mortar with gradual addition of small amounts of warm distilled water to a total volume to 200 ml. This requires 30 to 45 minutes, after which the solution is cooled to room temperature, filtered, and 4 drops of 3 per cent H_2O_2 is added. It may be stored at room temperature in a brown bottle for periods up to 6 months.

Procedure.—1. An air-dried blood smear is covered with the formalin-ethanol mixture for 5 seconds and gently washed with tap water for one minute.

2. The blood smear is then covered for 30 minutes with "working" Giemsa's stain which is prepared by the addition of 2 to 3 drops of Giemsa's solution per milliliter of phosphate buffer. The stain is *poured* off. Water should *not* be used to wash the stain from the slide.

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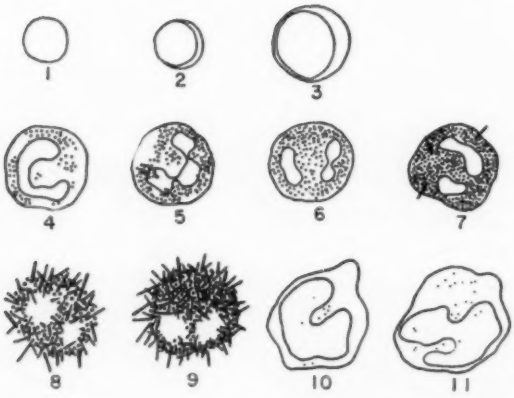

Fig. 1.—Schematic representation of peroxidase activity in blood cells. The dark dots and rods represent peroxidase-positive granules. 1, Red blood cell; 2, small lymphocyte; 3, large lymphocyte (1 to 3, peroxidase negative); 4 to 7, neutrophils; 8 to 9, eosinophils; 10 to 11, monocytes.

Fig. 2.

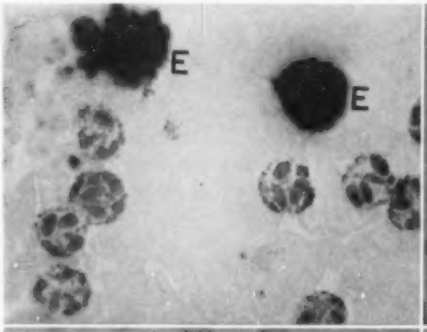

Fig. 3.

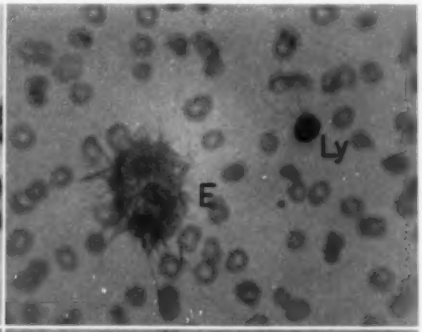

Fig. 4.

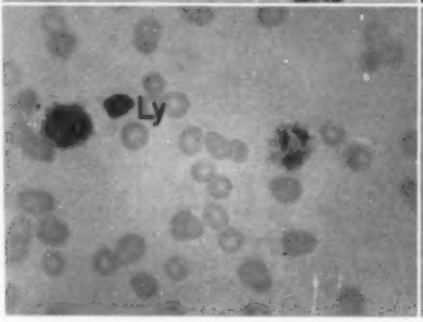

Fig. 5.

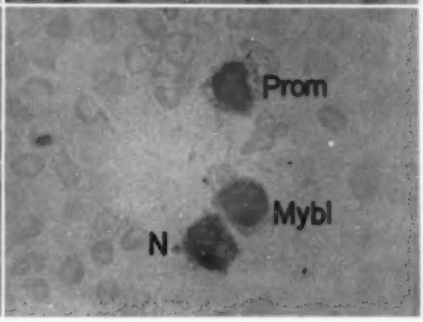

Fig. 2.—Two strongly positive eosinophils (E) and 7 moderately positive neutrophils. The nucleus of the eosinophils cannot be seen because of abundance of positive granules. Blood smear from normal subject. Peroxidase reaction.

Fig. 3.—One typical strongly positive eosinophil (E) and one negative small lymphocyte (Ly). Blood smear from normal subject. Peroxidase reaction.

Fig. 4.—Two positive neutrophils and one negative small lymphocyte (Ly). Blood smear from normal subject. Peroxidase reaction.

Fig. 5.—One questionably positive myeloblast (Mybl), one weakly positive neutrophilic promyelocyte (Prom), and one positive segmented neutrophil (N). Blood smear from a patient with acute myeloid leukemia. Peroxidase reaction.

3. The slide is flooded with the $CuSO_4$ solution for not more than 5 seconds which is then poured off, leaving a wet surface.

4. Benzidine solution at approximately 25° C. is placed on the slide for 2 minutes; the preparation is then washed with tap water and air-dried. (The pH of tap water is not significant here.)

The time for fixation must not exceed 5 seconds, since further fixation results in a reduction of peroxidase activity. Because of decolorization of the working Giemsa's stain by the $CuSO_4$ solution, this dye is used in higher concentration than usual. Since hydrogen peroxide undergoes deterioration, it may be necessary to add a small amount of fresh hydrogen peroxide to the benzidine solution before use.

Fig. 6. Fig. 7.

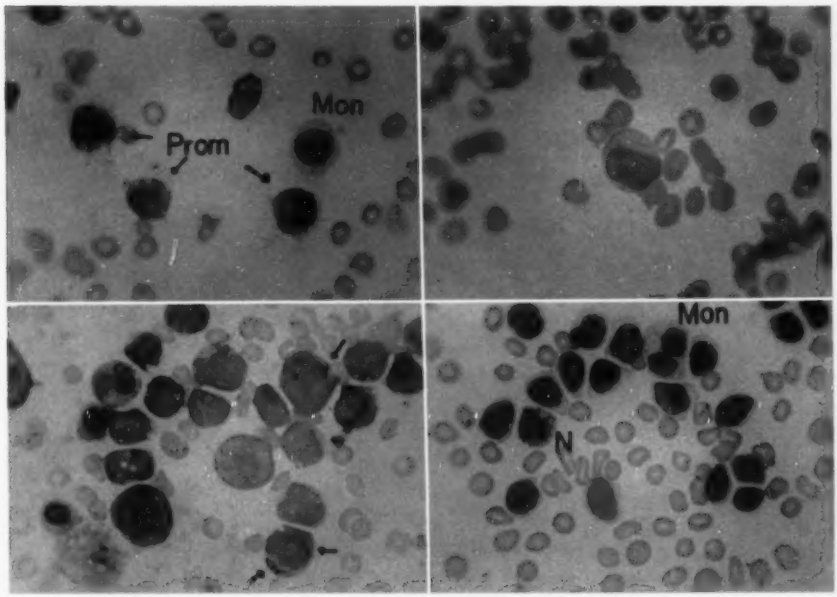

Fig. 8. Fig. 9.

Fig. 6.—Three positive promyelocytes (Prom) and one weakly positive monocyte (Mon). Blood smear from a patient with acute myeloid leukemia. Peroxidase reaction.

Fig. 7.—One myeloblast with Auer's bodies. Blood smear from a patient with acute myeloblastic leukemia. Wright's stain.

Fig. 8.—Strongly positive Auer's bodies (↑) in myeloblasts. Bone marrow smear from the same patient as in Fig. 7. Peroxidase reaction.

Fig. 9.—Many negative lymphocytes, one positive neutrophil (N) and one weakly positive monocyte (Mon). Blood smear from a patient with chronic lymphocytic leukemia. Peroxidase reaction.

RESULTS

Weak or moderate peroxidase activity is characterized by vivid green to dark-blue granules in the cytoplasm. With stronger activity, as seen in some neutrophils and in all eosinophils, there are rods and needlelike projections radiating from the cell margins which partially conceal the nuclei (Figs. 1 to 4). The cellular morphology is essentially unaltered from that seen in

the usual Giemsa stained preparation. The basophil usually fails to exhibit peroxidase activity. Monocytes, as shown on Figs. 1, 6, and 9, exhibit a few small peroxidase-positive granules, whereas lymphocytes and erythroblasts fail to show activity. (See Figs. 1 to 9.)

DISCUSSION

The method described consists of the adaptation of several features previously utilized by other workers and includes Graham's fixation method,[3] Giemsa's stain as the counterstain, and Sato-Sekiya's method[4] for the development of the peroxidase reaction. In the method herein described, the order of Giemsa's stain and the peroxidase development has been reversed. The advantages of the method include: (1) the blue-green color imparted to the peroxidase-positive granules rather than the yellow-brown color developed by the method of Graham; (2) the preservation of the morphologic nuclear and cytoplasmic detail of leukocytes, erythrocytes, and platelets; and (3) a relatively permanent and stable preparation.

In 1918, Graham described his method for the demonstration of leukocytic peroxidase activity. This method employed the use of formalin and alcohol fixation, benzidine for the tinctorial expression of the enzymatic activity, and hydrogen peroxide as the enzyme substrate. Disadvantages of Graham's method include variation in the color of positive granules which is proportional to the amount of benzidine, difficulty in distinction of the brown granules from other components of the cellular cytoplasm, and the instability of certain reagents in the test. McJunkin[5] modified Graham's method by the use of benzidine and Wright's stain in a phosphate buffer of pH 6.4. Shortly after McJunkin's method was published, Sato and Sekiya described their modification of the technique wherein alcohol-formalin fixation was discarded and a dilute solution of $CuSO_4$ was used to catalyze the reaction as well as for lysis of the erythrocytes. A nuclear counterstain of carbol fuchsin or safranin was used to provide morphologic detail and contrast between the nuclear material and cytoplasmic granules. Advantages of their technique include simplicity of the procedure, reagent stability, and relative permanence of the preparation. In 1934, Sato, Susuki, and Shibata[6] made a minor modification of this technique (increase of the concentration of $CuSO_4$) which facilitated hemolysis of erythrocytes. Beacom[7] described a modification of Goodpasture's technique[8] in which sodium nitroprusside was employed rather than $CuSO_4$ to produce a blue-green coloration in the presence of peroxidase activity.

Although Sato and Susuki have emphasized that differential counts of leukocytes may be performed on peripheral blood smears prepared by their method, it has been difficult or impossible to perform accurate differential counts on the bone marrow smears prepared by their technique. Because of this disadvantage, combinations of peroxidase and Giemsa's stains have been utilized in the author's procedure. Hirschfeld[9] described a method in which concentrated Giemsa's stain was used for short staining periods rather than

the methylene blue counterstain originally recommended by Graham. In 1928, Tokue[10] used Giemsa's solution for counterstaining and, shortly thereafter, Brinnitzer[11] described and utilized essentially the same method. Osgood[12] modified Goodpasture's method by the use of Giemsa's stain as a counterstain. This technique has been widely utilized, but morphologic nuclear detail is lost in this method. The method herewith described has been found to afford more constant tinctorial evidence of peroxidase activity in the leukocytes and to provide good morphologic detail of both leukocytes and erythrocytes.

The practical value and importance of the peroxidase stain in the diagnosis and therapy of hematologic disorders is a controversial issue. The method was utilized by Naegeli[13] and others to differentiate the myleoid from the lymphatic series of cells. The critical studies by Dunn[14] and by Jochman and Bluhdorn[15] clearly demonstrated that myeloblasts infrequently exhibit peroxidase-positive granules and, thus, that the technique is not of value in the differentiation of myeloblasts from lymphoblasts. Rosenthal[16] failed to demonstrate peroxidase activity in myeloblasts either from normal bone marrow or from patients with acute leukemia. In these same cells there was an absence of azurophilic granules. Thus, it would appear that the peroxidase staining method affords no help in the differentiation between myeloblasts and lymphoblasts. However, we have observed instances wherein the peroxidase stain has afforded better visualization of fine azurophilic granules which are seen only with difficulty and easily missed by Giemsa's or Wright's stain (Figs. 5 and 6). Auer's bodies show a strongly positive peroxidase reaction and are readily demonstrated with the peroxidase stain, whereas they may be less well visualized with conventional staining techniques (Figs. 7 and 8).

Furthermore, quite conclusive differentiation between monocytes and lymphocytes is afforded by the peroxidase stain, since the large majority of monocytes exhibit definite peroxidase activity which is visualized easily but less intensely than that seen in the granulocytes. This distinction is of value in patients who exhibit large lymphocytes in lymphocytosis or monocytosis during the course of certain viral, rickettsial, and other diseases.

SUMMARY

A convenient, rapid method for the peroxidase reaction combined with Giemsa's counterstain is described.

After rapid fixation with formalin-ethanol, relatively concentrated Giemsa's solution is used for counterstaining and then, according to Sato-Sekiya's method, copper peroxidase staining is effected. As a result, peroxidase-positive granules are seen as green to dark-blue in the cytoplasm. The usual cellular structures are well preserved by Giemsa's counterstain, so that each cell may be easily recognized. Finally, emphasis is placed on conclusive differentiation between monocytes and lymphocytes and on the diagnosis of some cases of acute myeloblastic leukemia with the peroxidase stain.

I wish to express my appreciation to Dr. Wallace N. Jensen, Assistant Professor of Medicine, and Dr. Theodore B. Rosenthal, Associate Professor of Anatomy, University of Pittsburgh, for their kind advice and encouragement.

REFERENCES

1. Winkler, F.: Der Nachweis von Oxidase in den Leukozyten mittels der Dimethylparaphenylendiamin-Alphanaphthol-Reaktion, Folia haemat. **4**: 323-328, 1904.
2. Schultze, W.: (a) Die Oxidaseraktion an Gewebsschnitten und ihre Bedeutung für die Pathologie (Zugleich ein Beitrag zur Differential-diagnose der Leukämieen), Beitr. path. Anat. u. allg. Path. **45**: 121, 1909.
   (b) Zur Differential diagnose der Leukämieen, München. med. Wchnschr. **56**: 167-169, 1909.
   (c) Weitere Mittelungen über Oxidasereaktion an Gewebsschnitten, München. med. Wchnschr. **57**: 2171-2173, 1910.
3. Graham, G. S.: Benzidine as a Peroxidase Reagent for Blood Smears and Tissues, J. Med. Research **39**: 15-24, 1918-1919.
4. Sato, A., and Sekiya, S.: A Simple Method for Differentiation of Myeloic and Lymphatic Leukocytes of the Human Blood, Tohoku Igaku Zasshi **6**: 534-541, 1922; Tohoku J. Exper. Med. **7**: 111-115, 1926.
5. McJunkin, F. A.: A Benzidine-Polychrome Stain for Blood, J. A. M. A. **74**: 17-19, 1920.
6. Sato, A., Susuki, T., and Shibata, Ry.: Normal Leukocytes, as They Appear Under the Copper Peroxidase Reaction, Tohoku J. Exper. Med. **24**: 195-201, 1934.
7. Beacom, D. N.: A Modification of Goodpasture's Technic for the Peroxidase Reaction in Blood Smears, J. LAB. & CLIN. MED. **11**: 1092-1093, 1926.
8. Goodpasture, E. W.: A Peroxidase Reaction With Sodium Nitroprusside and Benzidine in Blood Smears and Tissues, J. LAB. & CLIN. MED. **4**: 442-444, 1919.
9. Hirschfeld, H.: Erfahrungen mit der Oxidase-und Peroxidase-reaction, Med. Klinik **20**: 249-250, 1924.
10. Tokue, K.: The Oxidase and the Peroxidase Reaction in Different Leukocytes of the Blood, Tohoku J. Exper. Med. **12**: 445-458, 1928-1929.
11. Brinnitzer, H. N.: Über eine Kombination der Peroxydasereaktion und Giemsafärbung im Blutausstrich, Folia haemat. **41**: 240-244, 1930.
12. Osgood, E. E.: Atlas of Hematology, San Francisco, 1938, Stasney Co.
13. Naegeli, O.: Blutkrankheiten und Blutdiagnostik, Berlin, 1931, Julius Springer.
14. Dunn, J. S.: The Use of the Oxidase Reaction in the Differentiation of Acute Leukemia, Quart. J. Med. **6**: 293-308, 1913.
15. Jochman, and Bluhdorn: Über akute Myeloblasten leukämie, Folia haemat. **12**: 181-194, 1911.
16. Rosenthal, N.: Studies on the Oxidase Reaction of the Cells in Normal and Leukemic Blood, Arch. Int. Med. **20**: 184-197, 1917.

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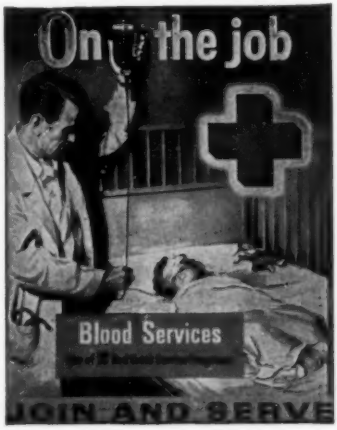

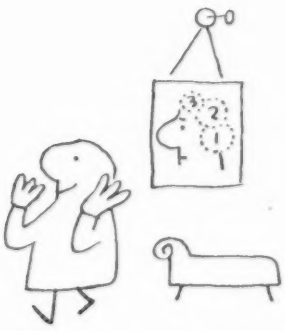

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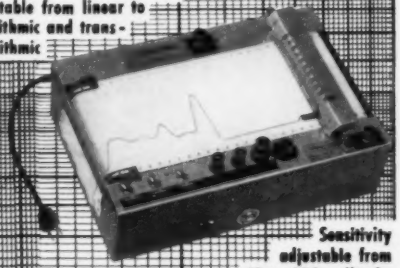

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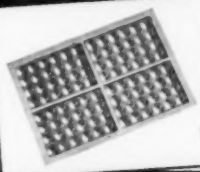

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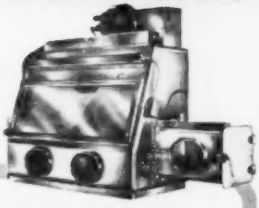

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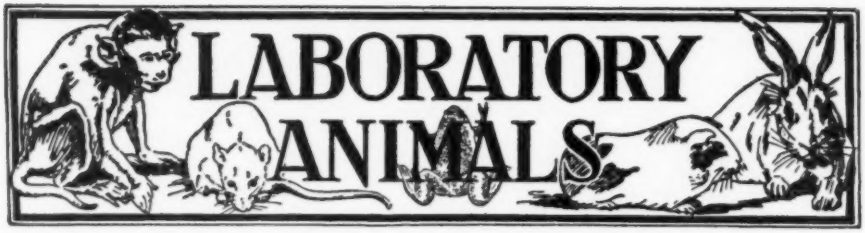

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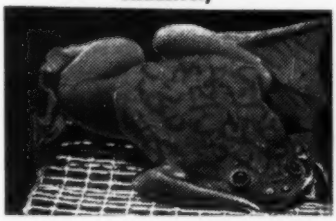

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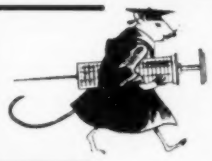

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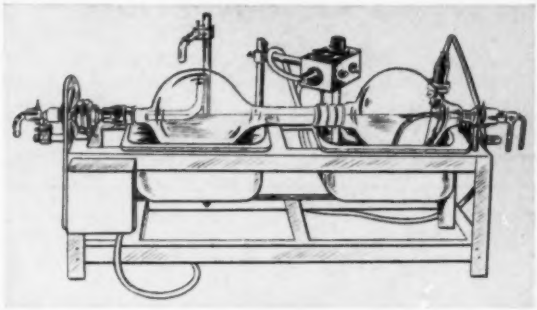

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